National Institute on Drug Abuse

RESEARCH

MONOGRAPH SERIES

Discovery of Novel Opioid Medications

147

U.S. Department of Health and Human Services • Public Health Service • National Institutes of Health

Discovery of Novel Opioid Medications

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NIDA Research Monograph 147
1995

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service
National Institutes of Health

National Institute on Drug Abuse
5600 Fishers Lane
Rockville, MD 20857

ACKNOWLEDGEMENT

This monograph is based on the papers from a technical review on "Discovery of Novel Opioid Medications" held on July 28-29, 1993. The review meeting was sponsored by the National Institute on Drug Abuse.

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National Institute on Drug Abuse
NIH Publication No. 95-3887
Printed 1995

NIDA Research Monographs are indexed in the *Index Medicus*. They are selectively included in the coverage of *American Statistics Index, Biosciences information Service, Chemical Abstracts, Current Contents, Psychological Abstracts,* and *Psychopharmacology Abstracts.*

Contents

Introduction

Rao S. Rapaka and Heinz Sorer

Heroin is an illegal and highly addictive narcotic. Addictive or dependence-producing properties are exhibited by (1) persistent regular use of a drug; (2) attempts to stop such use that lead to significant and painful withdrawal symptoms; (3) continued use despite damaging physical or psychological problems, or both; (4) compulsive drug-seeking behavior; and (5) need for increasing doses of the drug.

Many health problems related to heroin use are caused by uncertain dosage levels (due to fluctuations in purity), use of unsterile equipment, contamination of heroin by cutting agents, or use of heroin in combination with other drugs such as alcohol or cocaine. Typical problems include skin abscesses, inflammation of the veins, serum hepatitis, and addiction with withdrawal symptoms.

Utilization of unsterile needles by multiple individuals (needle sharing) increases the risk of exposure to the human immunodeficiency virus, the causative agent for Acquired Immune Deficiency Syndrome (AIDS). Heroin itself, as well as a drug-abusing lifestyle, may depress the body's ability to withstand infection.

While intravenous drug users account for approximately 25 percent of all reported AIDS cases, their proportion of the AIDS population appears to be increasing. In the first half of 1985, intravenous drug users accounted for 33 percent of all new AIDS cases. Moreover, 54 percent of newborns contracting AIDS have a parent who is an intravenous drug user, and intravenous drug users account for a similarly disproportionate share of the percentage of heterosexually transmitted AIDS cases.

Estimates of heroin use from the National Institute on Drug Abuse (NIDA) Household Survey are considered very conservative due to the probable undercoverage of the population of heroin users. Estimates of lifetime heroin prevalence have fluctuated from 2 million users in 1985 to 2.7 million users in 1991, 1.8 million users in 1992, and 2.3 million users in 1993. No significant changes in past-year prevalence have been detected.

An estimated 1.3 percent of the U.S. civilian, noninstitutionalized population aged 12 and older had "ever used" heroin. Among the age groups, lifetime use was most common for adults aged 26 to 34 (1.8 percent) and among older adults aged 35 or older who were unemployed (7.5 percent). There were some statistically significant differences among demographic groups, but they should be interpreted cautiously because of the small number of users. Among the total population, males were significantly more likely than females ($p < .001$), college graduates were significantly less likely than those with less education ($p > .01$), and those unemployed were significantly more likely than those in the other employment categories ($p < .05$) to have ever used heroin. Heroin use differed little by race/ethnicity, population density, or region, and there were few statistically significant differences within the age groups.

Because the rate of heroin use in the past year was 0.2 percent for the total population, no table is presented here for past-year use. Rates of use in the past month were so low among members of the target population that it was not possible to estimate them reliably using data from the 1991 NIDA Household Survey.

A household survey such as the NIDA Household Survey may yield conservative estimates of the extent of drug use among members of the general population, particularly for rarely used drugs such as heroin. Although, in 1991, the NIDA Household Survey included individuals living in some types of group quarters (i.e., homeless shelters, college dormitories, and rooming houses), those living in many other types of nonhousehold arrangements (e.g., homeless people living on the streets or sentenced criminals in correctional institutions) were not included in the survey. To the extent that heroin users are disproportionately represented in the populations not included in the NIDA Household Survey, the 1991 survey (even with the inclusion of individuals in some group quarters) likely underestimates the prevalence of heroin use in the general population.

In the 1992 NIDA Household Survey Populations Estimates Report, 0.9 percent of the civilian, noninstitutionalized U.S. population 12 years of age and older reported having used heroin in their lifetime, and 0.2 percent reported using it in the past year. In terms of population estimates, this amounts to 1.84 million persons for lifetime use and 323,000 persons for past-year use.

One of the provisions of the Anti-Drug Abuse Act of 1988 consists of establishment of a new division within NIDA, the Medications Development Division, which became official in 1990. Its goals include:

- Conducting necessary studies to identify, develop, and obtain Food and Drug Administration marketing approval for new medications for the treatment of drug addiction and other brain and behavioral disorders;

- Developing and administering a national program of basic and clinical pharmaceutical research designed to develop innovative biological and pharmacological treatment approaches for addictive disorders; and

- Establishing a close working relationship with pharmaceutical and chemical companies in the United States and abroad and with medications development programs in other agencies in the United States and abroad.

In late 1992, the Medications Development Division developed a preclinical program that has as its goal the discovery of potential new medications for improved treatment of opioid abuse and dependence. The Opioid Treatment Discovery Program currently is focused upon two clinical needs: (1) improved nonopioid medications for treating opioid withdrawal symptoms, and (2) adjunct medications for converting patients from opioid agonist therapy to opioid antagonist or nonopioid use (e.g., following medically facilitated withdrawal).

A need exists for improved medications with reduced abuse liability, reduced side effects, or longer duration of action than currently available opioid pharmacotherapies. There also is a need for medications that address the unique clinical needs that accompany opioid addiction in pregnancy.

This technical review was conceived with these goals in mind. The purpose of this technical review is to obtain up-to-date information regarding potential "second-generation" pharmacotherapies for opioid abuse and to stimulate new research regarding this chronic public health problem.

Targeting Drugs to the Brain by Sequential Metabolism

Nicholas Bodor

INTRODUCTION

The endothelial cells in the brain capillaries are joined very tightly, lacking fenestrae and, due to this reason, intracellular or transcellular transport of blood-borne compounds, including drugs, must take place directly through these endothelial cell membranes in order to reach the central nervous system (CNS). Due to their structure and nature, the brain capillary walls serve as a very effective barrier for the protection of the brain, and they commonly are referred to as the blood-brain barrier (BBB) (Brightman and Reese 1969). The BBB exhibits a particularly low permeability to hydrophilic compounds, such as polar molecules and small ions, which do not have specific transport mechanisms. It is important to emphasize, however, that the BBB appears to function as a physical barrier from both sides. Thus, hydrophilic molecules, which cannot easily penetrate the BBB if introduced to the brain generally by synthesis in situ, such as the various neurotransmitters, cannot and will not exit easily from the brain. The transport of various molecules through the BBB is hindered further by existence of various enzymes within the barrier and, thus, metabolically unstable substances may be degraded rapidly and do not reach the brain tissue intact (Brownlees and Williams 1993).

Peptide molecules, next to large proteins, present the ultimate challenge to be transported across the BBB. Although transport of some endogenous peptides through the BBB cannot be ruled out, it is very unlikely that physiologically significant amounts can reach the brain. Of course, in the circumventricular organs, the cellular elements of the tissue can be reached by the peptides. However, transport to deeper layers cannot take place. This, of course, is due to their hydrophilic nature but also because of the demonstrated high enzymatic instability (Powell 1993). The existence of transport systems for dipeptides and tripeptides were shown (Yamaguchi et al. 1970). A number of larger peptides, such as IGF-I, IGF-II, insulin, and transferrin are known to have receptors on the BBB. These receptors were identified on the luminal surface of the brain capillaries, and they are expected to be present on the antiluminal

borders since it is believed that these act as transcytosis systems (Pardridge 1986). An uptake and presumably transport system for the natural Leu-enkephalin on the luminal side of the BBB was identified (Zlokovic et al. 1989). However, the most important characteristic of an active (carrier-mediated) transport system is its saturability. In addition, it still is quite possible that this peptide will be metabolized during the next steps of the transport. This metabolism could take place in some of the compartments in parallel involving cytosolic endothelial space, luminal surface of the BBB, glial-end foot layer in apposition with the antiluminal side of capillary endothelium, and enkephalinergic synaptic regions juxtaposed to the brain microvessels (Zlokovic et al. 1988). It is well known that BBB enzymes recognize and rapidly degrade most naturally occurring neuropeptides (Brownlees and Williams 1993; Pardridge 1991). In view of these facts, the strategy involving design of enzymatically stable peptides, peptide analogs, or peptidomimetic drugs should and is being followed. However, the well-known physical characteristics of the BBB and the bidirectional nature of the passive transport still are presenting major problems for targeting peptides to the brain.

Here, a clear separation must be made of the concepts of *delivery versus targeting*. Of course, in the case of peptides, even delivery is a major problem. But *targeting means differential delivery*. The objective for neuropeptides would be to deliver the peptides preferentially to the brain and not the peripheral circulatory system. This, in principle, would allow longer-acting peptides in the CNS and avoidance of any peripheral activity.

Some of the strategies to deliver peptides into the brain were classified by Pardridge (1986) into three categories: (1) invasive procedures, (2) physiologically based strategies, and (3) pharmacologically based strategies. The most obvious invasive strategy is the use of an implanted intraventricular catheter, followed by infusion into the ventricular compartment. It is believed that this is the most direct and best way to deliver peptides to the brain. However, for most peptides this would allow only for delivery to the surface of the brain. Considering the deep convections of the human brain surface, most regions of the brain are no more than 0.5-l cm away from the ependymal surface or the cortical surface of the cerebrospinal fluid (CSF) compartment. Considering an average diffusion coefficient ($2x10^{-6}$ cm^2s^{-1}) for a peptide of 2,000-5,000 Da molecular weight, the effective diffusion distance of the substance is less than 1 mm (Poplack et al. 1981) if the metabolic rate is rapid, which

is the case for most known peptides having a $t_{1/2} < 10$ minutes (Powell 1993).

The other well-known invasive strategy is based on infusion into the carotid artery of a high concentration (> 1 M) of osmotically active substances such as mannitol or arabinose. Due to their osmotic activity, the brain capillary endothelial cells would shrink, resulting in transient opening of the tight junctions (Neuwelt and Rapoport 1984) that would facilitate the inflow of molecules that otherwise cannot cross the BBB. This heroic method is of limited use, however, due to the considerable toxic effects of the procedure. It many times can lead to inflammation, encephalitis, and the incidence of seizures (as high as 20 percent of the applications) due to the nonspecific nature of this delivery method. Indiscriminate delivery of many molecules from the blood can occur. In conclusion, these invasive procedures are justified only to be used in some life-threatening conditions where there is no other potential solution.

The physiologically based strategies involve the use of chimeric peptides (Pardridge 1985). This method is based on the assumption that some large peptides, which have receptors on the luminal side of the BBB (such as insulin, transferrin, or IGF-II), will be transcytosed, and they could carry some covalently bonded peptides. It was claimed, for example, that ß-endorphin coupled to insulin (Pardridge et al. 1990*a*) via a disulfide bond undergoes receptor-mediated transcytosis carrying the active peptide into the CNS. The active peptide, however, could not be detected in the brain. It appears that minimal if any transcytosis of the intact chimeric peptide takes place or that the speculated apparent cleavage of the active peptide from the conjugate effectively does not take place. Recently, antibodies to capillary endothelial cells were conjugated with peptides and claimed to result in trancytosis of the conjugate at the brain capillary level. So-called cationized albumins and cationized liposomes (excess basic amine group containing conjugates protonated to provide the cationization) also have been claimed to carry peptides to the brain (Pardridge 1991), but no pharmacological evaluation demonstrating the effectiveness of this method has been reported.

There are a number of serious drawbacks to the physiologically based approach. First, the poor stoichiometry of the neuropeptide to the carrier molecule limits the transport of the target peptide (a 500 to 4,000-Da peptide is loaded onto a 5,000 to over 100,000-Da carrier molecule). In addition, any carrier or receptor-mediated cellular transport has

physiologically severely limited transport capacity (saturable), thus limiting pharmacologically significant amounts from entering the brain. Thirdly, most of these carriers are not brain specific, as uptake by nonneural cells or by cells outside the CNS has been revealed (Ito et al. 1984). Finally, release of the active peptide from the conjugates has not been documented.

The objective of the pharmacologically based strategies is to convert water-soluble molecules into lipid-soluble ones. It was found that hydrophobic substances, because of their high lipid solubility, generally can diffuse in and out of the brain. Encapsulation of peptides in liposomes, which were shown to be taken up by cells lining the reticuloendothelial system of the liver and spleen, resulted in no measurable transport across the BBB (Pate1 1984). The use of prodrugs, that is, peptide latentiation by lipidization, was not successful. Both the C- and N-terminal hydrophilic groups in principle can be converted to the much more lipid-soluble diketopiperazine derivatives. This can be used only for small molecules like a thyrotropin-releasing hormone (TRH) (Hoffman et al. 1977), although even for small peptides the conversion of the diketopiperazines to the active peptide is questionable. It is clear that lipid solubility is not the sole determining factor in the passage of the intact peptide to the brain. Converting the carboxyl terminal to small esters is not enough, as these cleave far too easily and generally do not prevent endopeptidase or action of other peptide-cleaving enzymes on the molecule. The same is true for converting N-terminal to amides. Interestingly, even in a nicely lipid-soluble cyclic peptide like cyclosporin, which has no free carboxyl or amino termini and no charged groups, and since, in addition, four of the amide nitrogens are methylated, the compound is fairly bioavailable through the oral route; however, its BBB transport is paradoxically low (Cefalu and Pardridge 1985). This is due partly to the observed peptide degradation (Begley et al. 1990), although a recent report (Begley 1992) has pointed out that this lipid-soluble peptide is transported in the blood in combination with erythrocytes, leukocytes, and plasma proteins, all of which account for over 90 percent of the drug and, thus, concentration of the free drug in the plasma is limited greatly. Since the carriers of cyclosporin are unable to cross the BBB, the drug apparently has no vehicle to transport it into the cerebral compartment. The choroid plexus is the only exception where there is no such restriction to the contact of the cyclosporin carriers with cells. The apparent presence of active transport carrying cyclosporin out of the brain also might be an important contribution. It also needs to be emphasized that lipid-soluble compounds that are able to cross the BBB

can maintain the necessary active concentrations in the CNS only if their blood concentrations are maintained at adequately high levels. In view of the extremely short half-lives of most peptides in the blood (Powell 1993), this criteria cannot be satisfied in the usual cases.

The concept of restricted transport through the BBB of large lipophilic molecules based on size exclusion (Pardridge et al. 1990*b*), suggesting that compounds with molecular weight greater than 1,000 Da cannot penetrate the BBB, appears to be unfounded. The size exclusion related to the transport through lipid membranes must be associated primarily with the molecular volume determined by the actual geometry of the molecule, the overall conformation, and the heteroatom content. For flexible molecules, the movement across the membranes actually should be assisted by the thermal fluctuation of the membrane lipid (Träuble 1971), even if the molecular weight exceeds 1,000 Da.

It became evident in the present research effort that solving just one of the problems, that is, lipophilicity, or enzymatic stability simply is not enough for brain targeting of peptides. Three major issues must be dealt with simultaneously: (1) passive transport of the molecule based on its favorable lipophilic properties; (2) enzymatic stability to prevent premature degradation; and (3) affecting the bidirectional movement through the BBB. This latter component is the key to brain targeting of peptides and many other molecules. There also are equally important subsequent steps to assuring the final peptide targeting. These involve various enzymatic conversion-activation processes. Thus, the author's approach is a complex physical-chemical-enzymatic approach, a strategy called *molecular packaging,* where the target peptide system is placed in a molecular environment and the peptide itself appears as a perturbation of a bulky nonpeptidic-looking molecule dominated by lipophilic-modifying groups that assure BBB penetration and also prevent recognition of the peptide by peptidases. The author also attaches a specific functional group that serves as a *targetor* providing retention in the CNS of the conjugate. Finally, the controlled release of the biologically active target peptide is achieved by predictable sequential metabolism (Bodor et al. 1992).

A CHEMICAL DELIVERY SYSTEM (CDS) FOR TARGETING PEPTIDES TO THE BRAIN

A chemical delivery system (CDS) is defined as a biologically inactive molecule obtained by formal modification of an active drug by attachment of two different types of moieties (Bodor 1992). The most important component is the *targetor moiety,* which by predictable enzymatic conversions provides a physical-chemical or other means for organ or site targeting of the conjugate. Additional moieties then are coupled to this conjugate to optimize its physical-chemical properties and pharmacokinetic behavior. The concept of the targeting based on a specific targetor unit basically differentiates a CDS from a conventional prodrug (Bodor and Kaminski 1987; Bundgaard 1985). In the case of brain targeting, the corresponding CDS exploited the unique architecture of the BBB. The principle was to use the impermeable nature of BBB to advantage, from the inside of the brain. Accordingly, the general brain-targeted CDS should be sufficiently lipophilic to allow the brain uptake. However, the obligatory subsequent step is an enzymatic conversion of the targetor moiety that will promote retention within the CNS. This step actually does not have to be brain specific. In actuality, if this conversion takes place everywhere in the body, the enhanced hydrophilic character of this targeted modified conjugate would accelerate elimination from the periphery. Finally, the intermediate should be further metabolized within the brain to the active component in a sustained manner.

A general system based on the above concept is shown in figure 1. Here, the brain targeting is accomplished by the dihydropyridine-pyridinium salt-type targetor, which in the reduced dihydropyridine form is lipophilic and also enhances BBB penetration, but most importantly can be converted by enzymatic oxidation to a charged water-soluble, lipid-insoluble quatemary salt (T^+). While in principle there could be many different kinds of physical-chemical-based targetor moieties, the trigonellinate ↔ 1,4-dihydrotrigonellinate redox pairs have proven to be the most useful. That is because it and some of its close analogs allow derivatization of almost any functional group in a drug molecule. Secondly, the original lipophilic dihydropyridine form will be oxidized quite easily to corresponding quatemary salt essentially everywhere in the body. The mechanism of this oxidation has been extensively examined, and it has been suggested to be analogous to the oxidation of 1,4-dihydronicotinamide adenine dinucleotide or nicotinamide adenine dinucleotide phosphate, co-enzymes associated with numerous oxidoreductases and cellular respiration (Hoek and Rydstrom 1988).

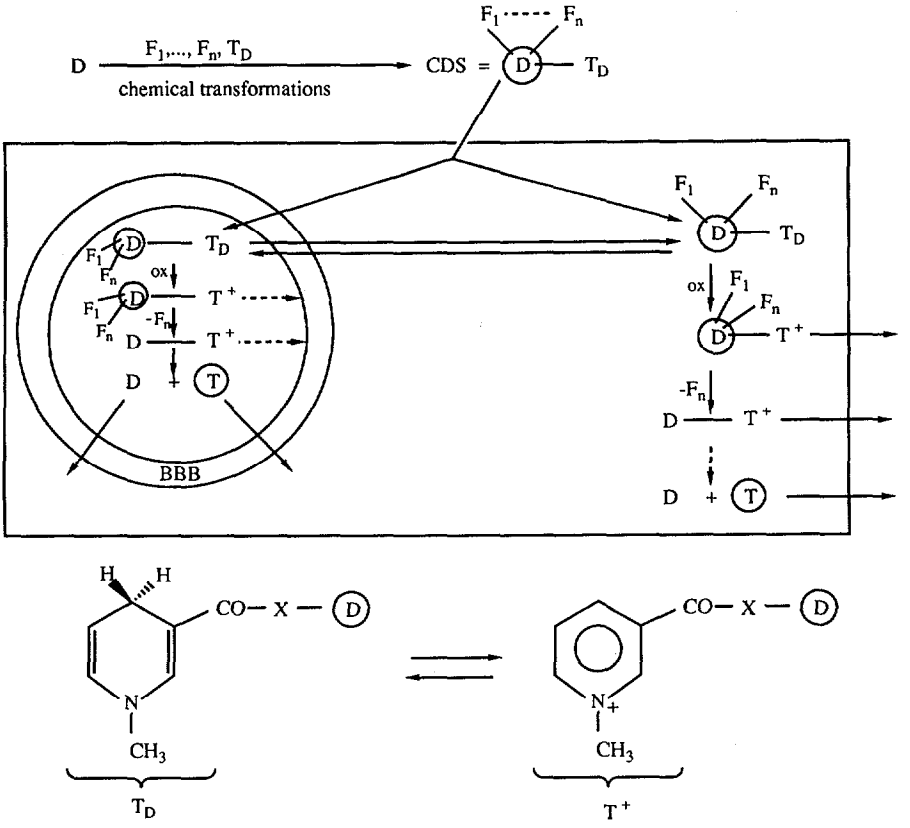

FIGURE 1. *Brain targeting by the CDS approach and the trigonellinate ↔ 1,4-dihydrotrigonellinate redox system*

KEY: F1...Fn = modifiers; T_D, T^+, T = different forms of the targetor; D = drug; CDS = chemical delivery system; OX = oxidation; X = O, NH, S

The process of oxidation of these molecules were shown to take place with direct hydride transfer without generating highly active or reactive radical type or other intermediates. This system was selected specifically to avoid any potential toxicity. Regardless of what the rest of the molecule is, placing a permanently positive charge on one end of the molecule is the most dramatic change one can introduce in a molecule's physical properties. The now polar-oxidized targetor-drug conjugate is trapped behind the lipoidal BBB and, in essence, remains "locked in" the CNS. The hydrophilic oxidized salt that is present in the periphery subsequently will be lost quite rapidly by elimination by the kidney and

bile. This will yield to a dramatic blood-brain ratio of this still inactive conjugate. In the next step(s), the trapped-in conjugate will undergo predictable enzymatic conversion, ultimately yielding the active drug. At the same time, the concentration of the active drug is very low in the periphery, mostly what can come out from the brain; thus, the dose-related peripheral toxicities will be reduced dramatically. Even in the CNS, the toxicity should be reduced since the trapped-in form is inactive. Consequently, the brain is not loaded by a peak concentration of an active component. The release of the active component depends on the rate of enzymatic reactions yielding the drug in the free form. This also should allow sustained release of highly potent compounds. This redox targetor concept has proved to be widely applicable for the brain targeting of a wide variety of substances (Bodor 1987; Bodor and Brewster 1983, 1991; Bodor and Simpkins 1983).

For peptides, in general, there are two potential sites for the attachment of the redox targetor moiety. The NH,-terminus would be the easiest to attach to, as in several other amino-containing compounds (Bodor and Brewster 1991), this approach resulted in successful brain targeting. Alternately, the carboxyl function could be derivatized using an acyloxyalkyl ester as the targetor moiety, a method successfully used for brain targeting of the peptide-related penicillins (Pop et al. 1989).

The author also has demonstrated that a peptide-like cyclic structure containing the redox targetor such as compound 1 can be delivered to the brain, followed by its oxidative conversion lock-in of compound 2 (see figures 1 and 2) (Bodor et al., unpublished results). This model compound contains peptide-like functions such as amides and the disulfide bond, and it is bicyclic. Thus, it appeared that, if a peptide CDS can be designed that would not be susceptible to peptidases in the BBB and would be lipophilic enough to get the brain, brain targeting of peptides should be successful (see figures 3 and 4).

The simple application of the redox targetor-based CDS to peptides, however, proves to be inadequate for brain targeting of peptides. The two main reasons are: (1) the lack of easy removal of the positively charged trigonelline amide from a peptide in terminal position, in addition to (2) the susceptibility of the peptide itself for various peptidase cleavage, while the targetor is still on the molecule. It also was found that using simple esterification on the molecule to protect the carboxyl

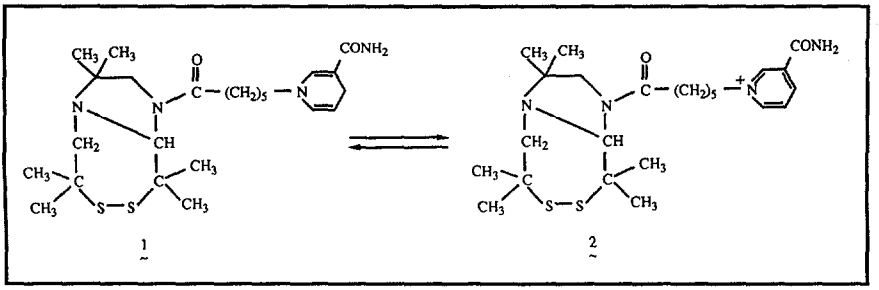

FIGURE 2. *Compounds 1 and 2*

function does not prevent its susceptibility to peptidase cleavage. Consequently, a general form (see figure 5) of a brain-targeted packaged peptide delivery system contains the following major components: the redox targetor (Tor), which is separated from the peptide by spacer (S) function(s) (strategically used amino acids extending the peptide); this assures aminopeptidase cleavage at the desired sites between the spacer amino acid and the target peptide. On the carboxyl terminal, however, it has been found that a bulky lipophilic moiety (L) is needed through an ester bond that substantially will increase the lipid solubility, and by its size and bulk, hinders this part of the molecule from being recognized by peptide-degrading enzymes. The first choice was cholesterol. Cholesterol esters of some amino acids and dipeptides have been prepared and were shown chemically to be sufficiently stable as a protective function (Shashoua et al. 1984). This part of the molecule, however, is labile toward esterase, lipase, or both, which permits its timely removal after delivery and trapping. In some cases, as it will be shown later, the carboxyl function of the peptide and the lipophilic function (L) need to be separated by another moiety, which at this point *can* be called the *C-terminal adjuster* (CA).

Having this highly packaged peptide, it is of utmost importance to have the designed enzymatic reaction take place in a specific sequence in order to achieve delivery of the active component. Upon delivery of the peptide delivery system, the first step must be the conversion of the targetor to charged functionality. This assures the lock-in. The next step must be removal of the large lipophilic function liberating a direct precursor of the peptide, which still is locked in due to the presence of the positively charged targetor. Subsequent cleavage of the targetor-spacer moiety then will lead to the release of the active peptide. Here, of course, the selection of the spacer function is very important, as its peptidolytic cleavage should be easier than peptidase fragmentation of the target

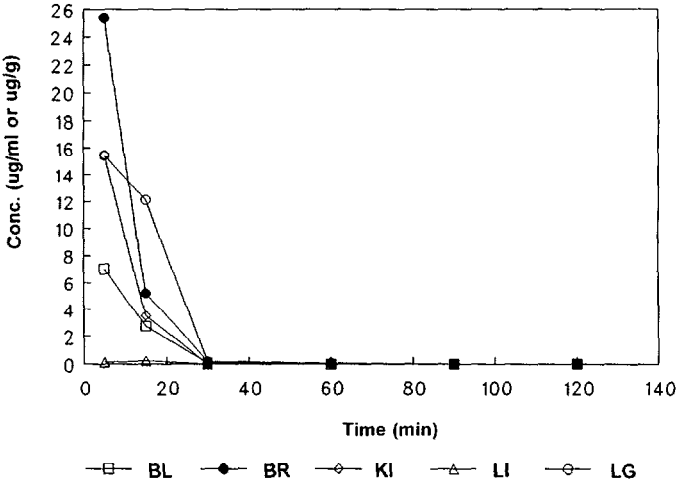

FIGURE 3. *Concentrations of compound 1 after i.v. administration (30 mg/kg) in rats*

KEY: BL = blood; BR = brain; KI = kidney; LI = liver; LG = lung

SOURCE: Bodor et al. (unpublished results)

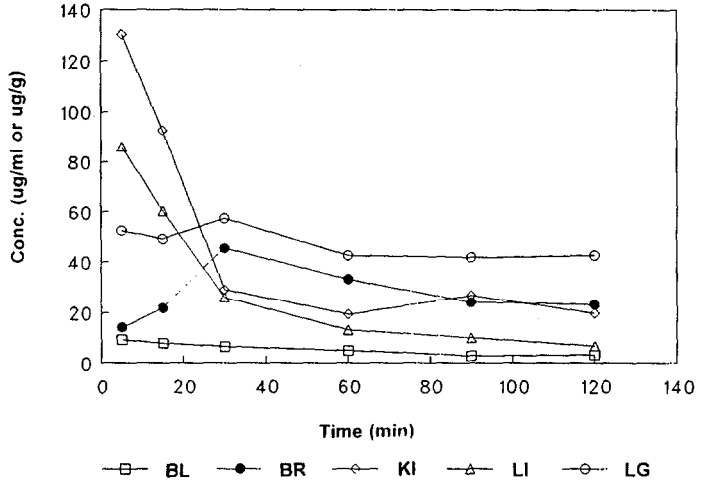

FIGURE 4. *Concentrations of compound 2 after i.v. administration of 1 (30 mg/kg) in rats*

SOURCE: Bodor et al. (unpublished results)

peptide part. All together, the architecture of the CDS for the peptide will fit into the general criteria of the peptide to be a perturbation on a bulky nonpeptide, like a nonpeptidase-sensitive lipophilic molecule terminated on the two sides by the lipophilic steroidal portion and the targetor, respectively.

To date, this molecular packaging-delivery system for peptides has been used for two important classes of neuropeptides: the synthetic analogs of opioid pentapeptide Leu-enkephalin, and TRH analogs.

TARGETING TO THE BRAIN OF LEU-ENKEPHALIN ANALOGS

The endorphins, enkephalins, and dynorphins are opioid peptides derived from glycoprotein precursors, such as proopiomelanocortin, proenkephalin, and prodynorphin, respectively. These endogenous peptides are part of an extensive neural network in both the central and peripheral nervous systems involving multiple ligands and multiple receptors. These peptides exert diverse physiological effects and exhibit complex pharmacology in mammals. Thus, studies have shown involvement of endogenous opioid peptides in the regulation of motor behavior (Sandyk 1985), seizure threshold (Frenk 1983; Olson et al. 1985; Tortella et al. 1981, 1985); immune responses (Plotnikoff et al. 1985; Wybran 1985); feeding and drinking (Baile et al. 1986); mental disorders (Frederickson and Geary 1982; Olson et al. 1985; Schmauss and Emrich 1985); and regulation of gastrointestinal (Olson et al. 1985; Porreca and Burks 1983; Schick and Schudziarra 1985), cardiovascular (Bernton et al. 1985; Holaday 1983; Johnson et al. 1985), neuroendocrine (Bicknell 1985; Grossman and Rees 1983; Millan and Herz 1985; Yen et al. 1985), and cognitive (Izquierdo and Netto 1985; Olson et al. 1985) functions. Their best-recognized central effect, however, is analgesia (Frederickson 1984). This particular activity has been observed mostly only after intracerebroventricular (i.c.v.) administration of the opioid peptides, while generally systemically administered compounds failed to exert CNS effect. This is only partly due to the metabolic instability of the endogenous opioid peptides, since most metabolically stable peptide analogs also lack analgesic activity after intravenous (i.v.) administration. Some of them showed some activity during peripheral administration, but the relationship between the analgesic doses after peripheral-versus-central administration was found to be highly unfavorable, suggesting that the entry of these analogs into the brain is quite sluggish, and very low CSF/plasma ratios were determined (Hill et al. 1981). It generally is

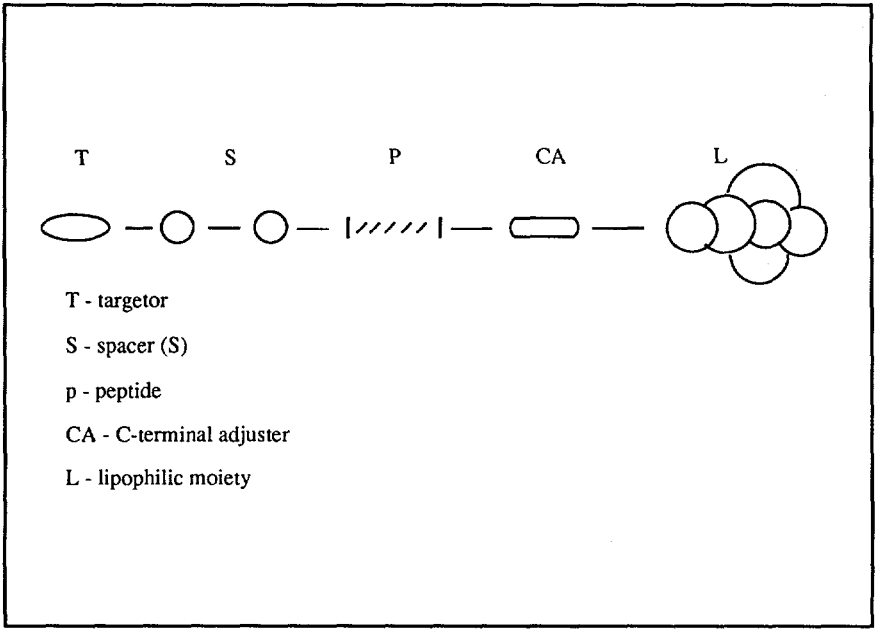

FIGURE 5. *General form of "packaged" peptide*

recognized that the capillary-bound BBB aminopeptidase activity is very high (Hersch et al. 1987; Pardridge and Mietus 1981; Pardridge et al. 1985), and possibly other "enkephalinases" also are present (McKelvy 1983).

According to the general form of the packaged peptide, a CDS has been designed for brain delivery of analogs of Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu, YGGFL) based on several considerations. This natural enkephalin is sensitive particularly to cleavage and, thus, to deactivation by endopeptidases at the end terminal tyrosine position and in the middle between Gly^3-Phe^4 position. The best way to prevent premature cleavage of the thyrosine is the replacement of the Gly^2 by D-Ala. At first the author thought that inclusion of the bulky lipophilic steroidal moiety (L) would give protection against potential cleavage located close to carboxyl-terminus. The brain targeting is achieved by placing the 1,4-dihydrotrigonellinate targetor (T) at the N-terminus via an amide-type covalent bond. Previous experience had shown, however, that trigonelline amides are quite stable and, thus, removal of the positively charged targetor would not be accomplished prior to aminopeptidase cleavage of the peptide part. In order to achieve the release of the peptide at the desired point of the sequence, the peptide and the targetor have

been separated by a spacer (S), which should facilitate the release of the peptide. Due to the involvement of alanine-aminopeptidase in the enkephalinergic transmission in brain (Schwartz et al. 1987), L-Ala was selected as a "spacer" as a first approximation. The packaged enkephalin analog is shown on figure, 6 as structure 3. Here, the YAGFL generically represents the structure regardless of what the configuration of the individual amino acids are. The scheme in figure 6 then shows the expected sequence of enzymatic metabolic events, which ultimately would lead to the release in the brain of the active enkephalin analog.

First, however, some relevant in vitro studies were performed to predict the expected biotransformation products. Studies were done in phosphate buffer, whole blood, and 20 percent (w/w) brain homogenate. As expected, the CDS (structure 3) is very unstable in biological fluids ($t_{1/2}$ is 1.28 minutes in rat blood, 4.0 minutes in rat brain homogenate). The quaternary intermediates to be formed (intermediates 4 and 5) were found to be stable in the pH range of 4-9 for at least 6 hours. However, intermediate 4 has a half-life of 2.24 minutes in whole blood and 37.84 minutes in brain homogenate, while $t_{1/2}$ of intermediate 5 is 101.7 and 3 1.26 minutes in blood and brain homogenate, respectively. These data indicate the desired possible transformation ability in vivo. Receptor-binding studies (competition with [^{3}H]-diprenorphine, a y-receptor agonist) confirmed that intermediate 4 has very low ($IC_{50} = 5.6x10^{-6}$ M) affinity, while intermediate 5 has slight ($IC_{50} = 2.0X10^{-7}$ M) affinity to opioid receptors, as compared to the parent peptide, [D-Ala^2]-Leu-enkephalin ($IC_{50} = 4.0X10^{-8}$ M). It can be concluded, therefore, that only a "true delivery" of the enkephalin analog would result in profound biological response.

For the in vivo studies, in order to avoid the possible pitfalls of using radiolabelled compounds, analytical procedures based on mass spectrometry were developed. It previously has been shown that electrospray ionization (ESI) and fast atom bombardment (FAB) mass spectrometry provide specific and sensitive methods to determine thermally labile quaternary trigonelline compounds (Prokai et al. 1989).

For in vivo studies in rats, the author wanted to avoid the brain-uptake index method, which is based on administration to the carotid artery, which highly favors even minimal brain delivery. It was chosen to administer the drug via i.v. injection to the tail vein. After the injection of the compound, the animals were sacrificed at various time intervals, and brain and blood samples were collected. The brain samples were

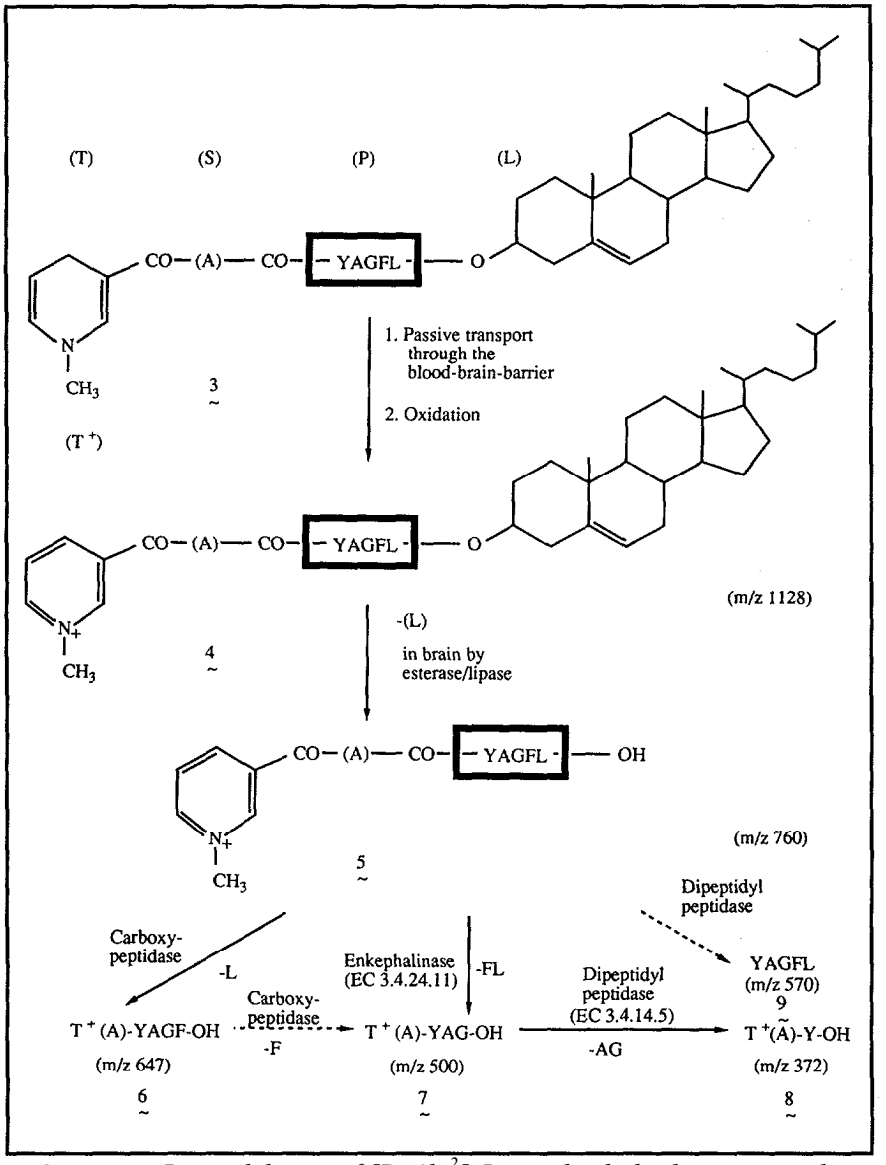

FIGURE 6. *Brain delivery of [D-Ala^2]-Leu-enkephalin by sequential metabolism*

homogenized and centrifuged, and the extracting supematant solution first was purified by chromatography. Subsequently, the effluent was directly analyzed by ESI mass spectrometry. The obtained ESI mass spectra (figure 7) clearly showed the presence of compound 5 (m/z 760),

which was absent from the analytical sample obtained from the brain of the animals treated with the vehicle only. An estimate of the amount of the locked-in compound 5 is about 500-700 pmol per gram of tissue 15 minutes after i.v. administration, by comparing the absolute intensity of m/z 760 to that of a sample obtained from the brain of an undosed animal, to which a known amount of compound 5 had been added. In tissue collected 1, 2, and 4 hours after systemic administration, compound 4 could no longer be identified, and the quantity of compound 5 proportionally (with $t_{1/2}$ of about 60 minutes) decreased with time. In none of the blood samples could these compounds be detected, indicating very effective destruction of them, most likely in the liver.

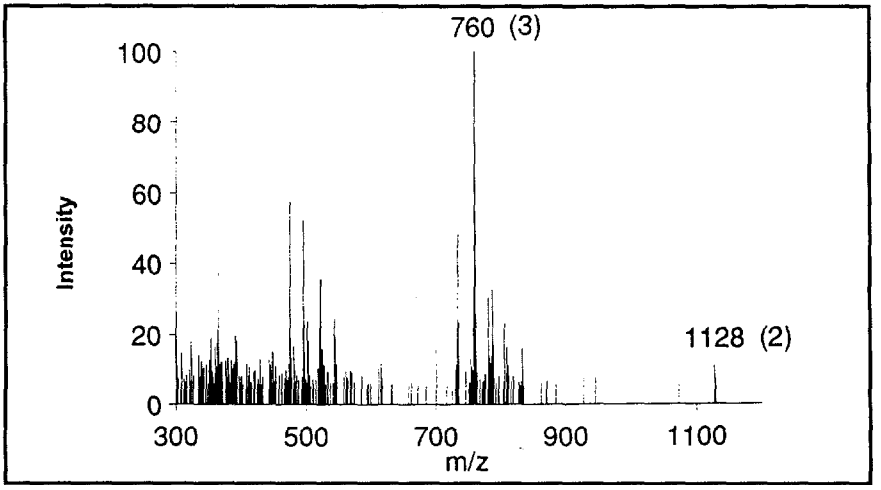

FIGURE 7. *ESI mass spectrum of the brain sample 15 min after the administration of the [D-Ala^2]-Leu-enkephalin CDS (compound 3)*

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The first locked-in intermediate compound 4 (m/z 1128) was detected in the samples at low amounts. Most likely this is due to very low recovery from the tissue by the sample work-up of this large lipophilic molecule. In addition, the technique would be much less sensitive towards compounds with lipophilic moiety. The design principles, however, relied on the facile removal of the protective ester function by enzymatic hydrolysis and, from the beginning, compound 5 was considered the key locked-in intermediate.

The results obtained were validated by FAB mass spectrometry. The trideuteromethyl analog of compound 5 was synthesized and used as a specific internal standard to estimate the quantity of intermediate compound 5. This internal standard has a distinctive m/z 763 ion, but there is no other difference in physicochemical properties from compound 5, detected at m/z 760. The mass spectra were collected by multichannel signal averaging over a narrow mass range (m/z 750 to 770). The high concentration (nearly nanomole/g tissue) and the sustained level (in vivo $t_{1/2}$ of 60 min) of the locked-in species that contains the intact peptide segment confirmed the findings with ESI spectrometry. Brain samples were analyzed similarly after administration of the parent enkephalin and its cholesterol ester in equimolar doses. No detectable amount of compounds with the peptide part intact was found. There were indications that fragments of the cholesterol-protected peptides penetrated the BBB, although they disappeared quickly from the brain. This is expected, as the aminopeptidase but possibly not the endopeptidase is able to sequentially remove the N-terminal amino acids of the molecule. It is evident that the targetor function is necessary to furnish stability and lock-in by virtue of the ionic trigonellinate moiety formed in situ within the brain. It seems that the cholesterol ester is cleaved rather easily after or during the transport through the BBB.

The key and most convincing evidence should come, however, from pharmacological studies. The analgesic effect of the CDS (compound 3) was determined by the method of tail flick latency response by animals injected with the vehicle solution, 10 mg/kg of (D-Ala^2)-Leu-enkephalin, and equimolar CDS (compound 3), respectively. The i.v. administration of the compound, indeed, has shown a tendency of increased analgesic activity, as compared to the parent peptide. However, the magnitude of the response remains much lower than would be expected from the amount of the determined locked-in quaternary peptide conjugate (compound 5). Since the amount of intermediate 5 after the delivery decreases profoundly, cleavage of it must take place at sites other than the desired removal of the targetor-spacer moiety. As a matter of fact, in vitro experimentation has demonstrated that the Ala-Tyr^1 peptidolysis is not predominant. As shown in figure 8, the neutral endopeptidase, EC 3.4.24.11, ("enkephalinase") is, as with the parent peptide, the major degrading enzyme resulting in an inactive species 7 (m/z 500) due to the cleavage of the Gly^3-Phe^4 bond.

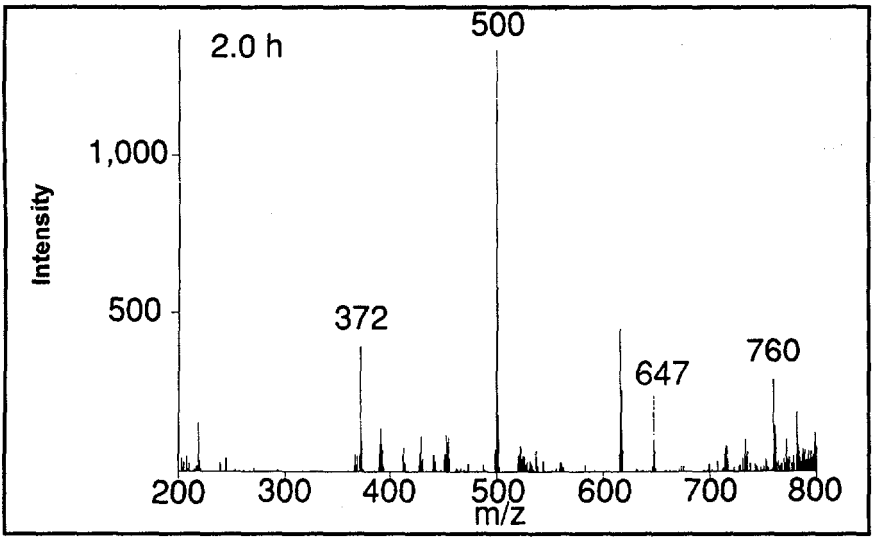

FIGURE 8. *ESI mass spectrum showing the cleavage of the locked-in T^{+}-Ala-Tyr-[D-Ala]-Gly-Phe-Leu (m/z 760) in vitro (brain homogenate, incubation at 37 °C for 1 hour)*

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Carboxypeptidase is another deactivating enzyme removing the C-terminal (L)-Leucine, resulting in compound 6 (m/z 647). The fragment 7 further is cleaved by dipeptidyl peptidase (EC 3.4.14.5), yielding the small targetor-dipeptide fragment 8 (m/z 372). The desired (D-Ala^2)-Leu-enkephalin could not be detected among the cleavage products of compound 5, although small amounts may be released from the targetor-peptide conjugate in the CNS in vivo, resulting in the measured analgesia.

It should be emphasized that the concentration of compound 5 cannot be used as a measure of the efficiency for peptide delivery. While clearly supporting the mechanism proposed, this compound is an intermediate in a process governed by sequential metabolism and the lock-in mechanism. Due to extraction difficulties, compounds 3 and 4 were not quantitated. However, the key intermediate compound 5, which is formed from compound 4 and then consumed in various processes outlined above, might be present in a quasi-steady state concentration as long as the precursors are present in the brain. As shown in figure 9, the concentration of compound 5 is determined by rate constance k_1, k_2, k_3, as well as k_4-k_n. It became evident, however, that the undesired degradation of compound 5 dominates the process. To circumvent this problem, the

terminal leucine residue was replaced by D-isomer. This modification, that is, the use of D-Ala-D-Leu enkephalin, also enhances the resistance to enkephalinase. As shown in figure 10, in the in vitro experiment in 20 percent brain homogenate, more than 90 percent of the targetor-DADL-enkephalin conjugate still is present with a single L-alanyl spacer intact after 4 hours of incubation. However, the presence of the corresponding protonated free enkephalin analog was shown by the presence of its ion at m/z 570. The unwanted peptidase cleavage fragmentations virtually ceased. The slow release at the site of action and subsequent slow degradation by specific peptidases may result in sustained release and a steady concentration of the biologically active peptide in the CNS.

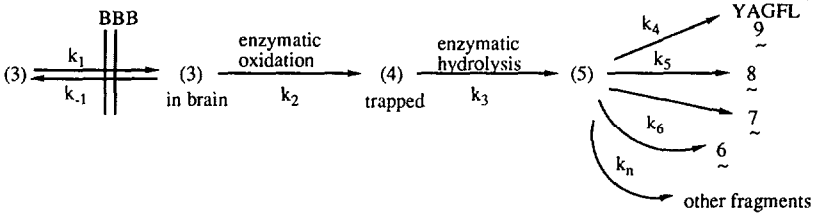

FIGURE 9. *Rate processes affecting the delivery of the enkephalin analog*

Indeed, for the CDS of the DADL-enkephalin, a long-lasting and statistically highly significant increase in the tail flick latency, a measure of the spinal cord-mediated analgesia of rats after systemic administration, was observed as shown in figure 11. The vehicle control, the unmanipulated DADL-enkephalin analog, and the partially conjugated peptides (either with the targetor-spacer *or* with the cholesterol) showed no effect. Importantly, very significant and sustained increase had been obtained even 5 hours after i.v. administration. This certainly is due to the specific and most important value of the CDS approach (i.e., the lock-in mechanism). The type of curve presented in figure 11 is distinct from those characteristic to simple delivery of the enkephalins directly to the brain by i.c.v. injection. It previously has been reported that, with a CDS for a molecule like estradiol, one can achieve therapeutic, sustained, and steady-state concentration in the brain, as well as subsequent pharmacological activity for up to 6 weeks after one single dose (Anderson et al. 1987).

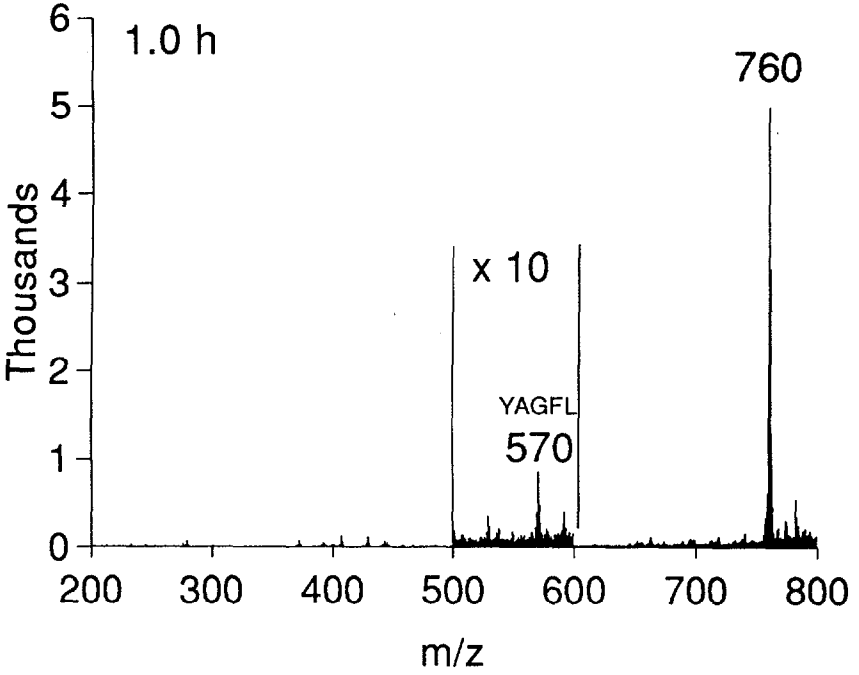

FIGURE 10. *ESI mass spectrum showing the cleavage of the T^{+}-Ala-Tyr-[D-Ala]-Gly-Phe-[D-Leu] (m/z 760) in vitro (brain homogenate, incubation at 37 °C for 2 hours)*

TARGETED BRAIN DELIVERY OF A CNS-ACTIVE TRH ANALOG

TRH is found in the median eminence at the highest concentration (Brownstein et al. 1974). Its primary role is on the secretion of the neurotrophic hormone thyroid-stimulating hormone (TSH) (Boler et al. 1969; Breese et al. 1975), but the extrahypothalamic distribution of TRH and its receptors indicates that TRH plays other roles in nervous system physiology.

The best-documented and most interesting extrahypothalamic effect of TRH is its analeptic action. Accordingly, high doses of TRH (up to 100 mg/kg) administered peripherally (Breese et al. 1975; Horita et al. 1976; Kraemer et al. 1976; Miyamota et al. 1982) and lower doses administered in a specific region of the brain (Horita et al. 1986; Kalivas and Horita 1980) have been shown to reduce pentobarbital-induced sleeping time by

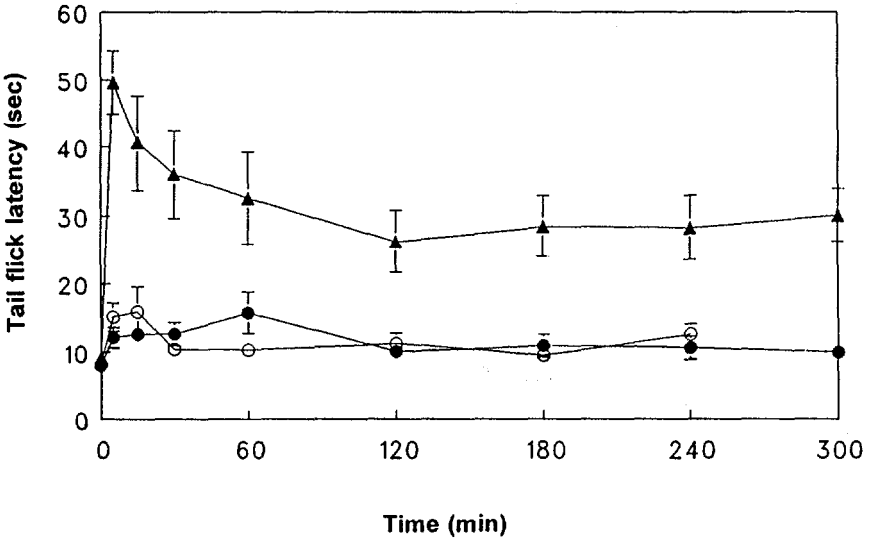

FIGURE 11. *CNS effect of the i.v. injection (8.8 µmoL!kg of body weight) of [D-Ala^2]-[D-Leu^5]-enkephalin CDS (▲), compared to that of the parent peptide (●) and of the vehicle solution (○) as a control. Measure of analgesia: tail flick latency on Sprague-Dawley rats*

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50 percent or more in rats, rabbits, and monkeys. This analeptic effect can be antagonized by the muscarinic receptor blockers scopolamine and atropine (Breese et al. 1975; Horita et al. 1976; Kraemer et al. 1976; Miyamota et al. 1982), indicating the involvement of a cholinergic mechanism. In normal animals, TRH has little effect on cholinergic neuronal activity (Yarbrough 1979). On the other hand, if the cholinergic activity is depressed in the animals, TRH has been shown to enhance activity. High-affinity choline uptake (Atweh et al. 1975) is reduced by pentobarbital, and TRH prevents this effect in the hippocampus, cortex, and midbrain (Schmidt 1977). Similarly, MK-721, a potent TRH analog (Porter et al. 1978), antagonizes the barbiturate-induced decrease in high-affinity choline uptake in the hippocampus and cortex (Santori and Schmidt 1980) when given i.c.v.

A recent report by Horita and colleagues (1989) suggests a potential cytoprotective role for TRH in rats. The researchers observed that the TRH analog MK-771 will reverse the reduction in hippocampal

computerized axial tomography activity and high-affinity choline uptake induced by ibotenic acid injection into the septum. They also have reported that the rate of learning in a 12-arm radial maze was enhanced in septal-lesioned rats with daily TRH treatment.

It also was reported that TRH administration showed positive effects on memory in patients with probable Alzheimer's disease (Lampe et al. 1991; Mellow et al. 1989).

Considering that TRH has an extremely short half-life (Bassiri and Utiger 1973) and it does not effectively penetrate the BBB (Szirtes et al. 1984), these few clinical evaluations are encouraging. Accordingly, molecularly packaged, brain-enhanced delivery and release of more stable analogs of TRH would have a potential use for therapy in Alzheimer's patients.

There have been numerous attempts to modify the structure of TRH in order to obtain metabolically stable analogs and to improve selectivity by dissociating the endocrine and CNS function. It was found that both these aims can be achieved by incorporating aliphatic amino acids in place of His^2 (Szirtes et al. 1984). Nevertheless, although these modifications improve metabolic instability and somewhat the poor lipid solubility of TRH, their peptide character still prevents significant brain delivery. Of course, the bidirectional transport of these molecules is not affected by these designed strategies for TRH analogs, so sustained brain delivery may not be accomplished.

The packaging strategies applied for the enkephalin analogs can be applied to neuropeptides with free NH_2- and -COOH termini. In TRH, however, no free hydroxy or amino groups are present. From the above outlined molecular packaging point of view, TRH (compound 10) presents three additional problems. Although it is a small tripeptide, the presence of the pyroglutamate function on the N-terminal, the prolinamide on the C-terminal and the histidine in the middle all presents additional problems. The problem of histidine is solved easily, as histidine actually is not essential for the CNS activity. In fact, the respective (Leu^2), (Nle^2), and (Nva^2) analogs have 2.5 to 10 times greater CNS activity in the inhibition of catalepsy, as compared to TRH (Szirtes et al. 1984). As shown in table 1, the (Leu^2) analog (compound 11) shows increased CNS activity and a dramatic decrease in TSH-releasing effect. On the other hand, further modifications on the molecule, that is, using the D-amino acids alternately in structure 11, as represented by structures 12-14 in table 1, would stabilize the molecule against peptidase

TABLE 1. *CNS and hormonal activities of TRH and [Leu^2]-TRH (79)*

Nr.	Compound	Anticataleptic effect (ED_{50}, mg/kg i.v.)	TSH-releasing effect (%)
10	pGlu-His-Pro-NH_2 (TRH)	113	100
11	pGlu-Leu-Pro-NH_2	40	2
12	D-pGlu-Leu-Pro-NH_2	70	0
13	pGlu-D-Leu-Pro-NH_2	>80	0
14	pGlu-Leu-D-Pro-NH_2	>80	0

cleavage. However, it was found at the same time that the activity is decreased significantly. In view of the packaging resulting in enhanced stability against amino peptidase, anyhow, there was no point in using any of the analogs 12-14, but structure 11 was selected for developing specific packaging strategies for it. A look at the TRH precursor consisting of 123 amino acids revealed that it contains 3 copies of a specific sequence containing the TRH progenitor flanked on each side by dibasic residues (Jackson 1989; Richter et al. 1984). The progenitor sequence QHPG indicates that glutamine, not glutamic acid, is the precursor for the N-terminal pyroglutamate, while the presence of the C-terminal glycine clearly functions as an amide donor for amidation of the proline (Bradbury et al. 1982). This process was shown (Fischer and Spiess 1987) to be catalyzed by the enzyme peptide glycine alpha-amidating monooxygenase (PAM), which requires Cu^{++}, ascorbic acid, and molecular oxygen. Accordingly, it has been considered that the progenitor sequence QLPG (compound 15) would be the right compound to be packaged in order to achieve central delivery of the TRH analog compound 11. Cyclization of the N-terminal glutamine is known to be catalyzed by a specific enzyme, glutaminyl cyclase (Fischer and Spiess 1987). Thus, the crucial step to the proposed strategy is that the corresponding locked-in targetor-spacer-linked progenitor, as represented by structure 16, is a substrate for the amidating enzyme PAM. This hypothetical locked-in precursor 16 was synthesized, and its biotransformation was studied in vitro in brain homogenate. The results obtained, as shown in figure 12, indicate that the conversion of the

precursor 16 (m/z 605) to the desired prolinamide derivative 17 (m/z 547) occurs faster than any other enzymatic cleavage reaction examined.

Thus, the delivery of TRH analog 11 depends on the release of the Gln-Leu-Pro-NH,, compounds 18 from 17 by dipeptidyl peptidase, similar to the enkephalin case. Pharmacological studies clearly indicate that the expected bioactivation to the TRH analog 11 in the CNS must take place. A profound decrease in the barbiturate-induced sleeping time, the measure of the activational effect on cholinergic neurons in mice, is shown in figure 13. At equimolar (30 μmole/kg) dose, the i.v. administration of the intact TRH analog 11 showed only marginal effect due to limited BBB penetration, while the CDS having one (L)-Ala spacer has resulted in approximately 30 percent reduction in the sleeping time.

The expected sequence of metabolic events is summarized in figure 14. As figure 14 and structures 15 and 11-18 previously exemplified on the enkephalin case in some general terms on figure 9, the relative rates of all these enzymatic transformations are crucial to achieve the ultimate delivery. As long as the target tripeptide is flanked between the targetor-spacer and the C-terminal adjuster (CA in the general packaged form) as in structure 16, it is not expected to undergo unwanted peptidase cleavage. However, after the facile conversion of the glycine terminal to the prolinamide, the targetor containing conjugate 17 might undergo removal of the proline by endopeptidase, yielding the T^{+}-AQL fragment (m/z 450), of which formation actually was shown in the in vitro experiment as demonstrated in figure 12. Consequently, some refinement has been considered, particularly in the spacer, to facilitate the conversion of compound 17 to the direct precursor 18. One Ala residue has been selected initially because of its simplicity and because it is a substrate also of the prolyl endopeptidase (Yoshimoto et al. 1978). However, the N-terminal of the spacer Ala is blocked by the targetor, which very much reduces its substrate properties. Therefore, the spacer part has been extended with another Ala. The pharmacological consequence of this modification, as shown on figure 13, resulted in more than 50 percent decrease of the barbiturate-induced sleeping time in mice.

CONCLUSIONS

The molecular packaging based on the redox targetor and cholesterol, refined by the spacer and C-terminal adjuster, has been the first rational

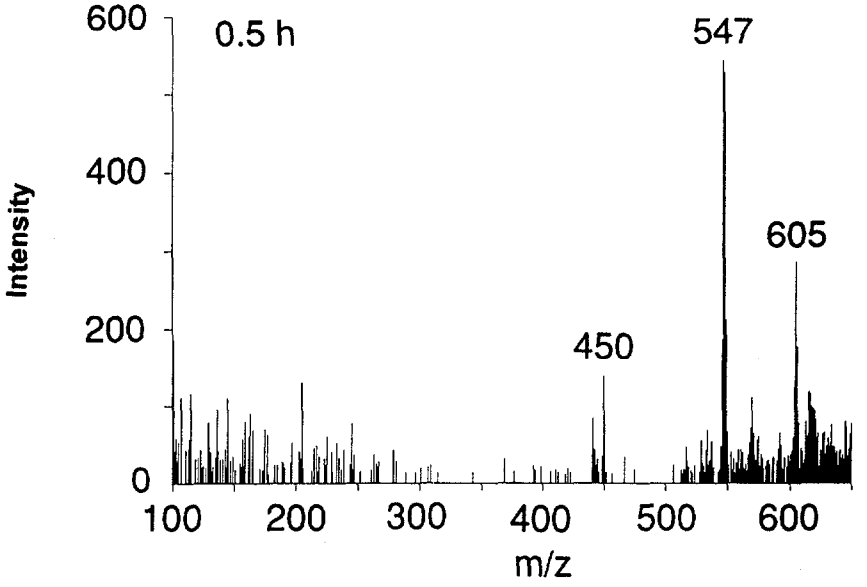

FIGURE 12. *ESI mass spectrum showing the cleavage of the T^{+}-Ala-Gln-Leu-Pro-Gly (m/z 605) in vitro (brain homogenate, incubation at 37°C for 0.5 hour)*

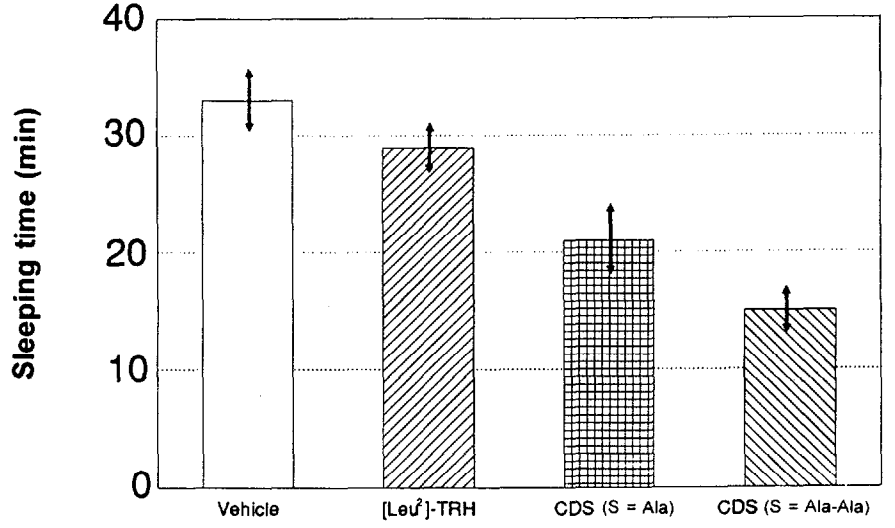

FIGURE 13. *Effect of the TRH analog pGlu-Leu-Pro-NH_2 and its CDSs to the methohexital-induced sleeping time in mice after i.v. injection (30 µmol/kg body weight dose)*

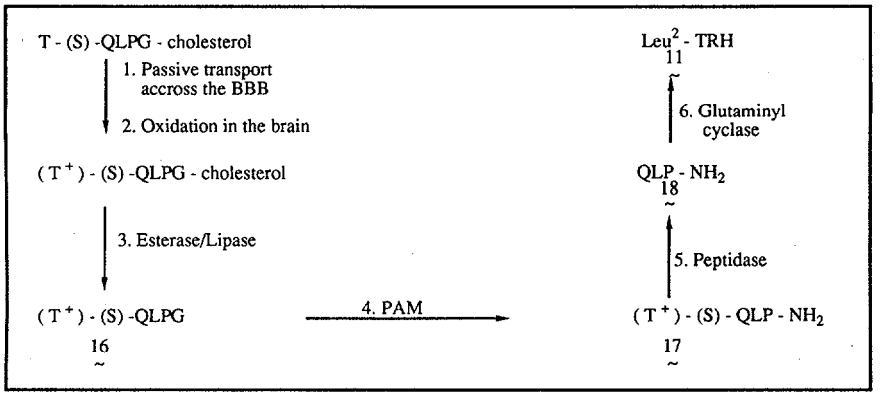

FIGURE 14. *CNS delivery of pGlu-Leu-Pro-NH_2 by sequential metabolism*

drug design approach for which the brain delivery of these important biomolecules in a pharmacologically significant amount has been documented by detailed mass spectrometric studies providing high levels of molecular specificity, in addition to demonstrating significant pharmacological response. The strategy emphasizes the importance of controlling bidirectional transport and metabolism in order to achieve targeting of peptides to the site of action. The two examples given demonstrate that, due to the complexity of the sequential metabolism required for success, each case has to be considered in detail, and the design process should fit the specific peptide. The overall strategy is based on structural, physical-chemical, and enzymatic aspects of the BBB with respect to the molecules transported through it, in addition to the designed sequential metabolic processes.

The already-studied CDSs possibly can be further refined but, more importantly, the general method should be extended to other classes of peptides in order to determine the scope and limitations of the method. Specific conclusions have to be drawn as to how the size, physical properties, and general complexity of the target peptide can influence the CNS delivery by sequential metabolism.

ACKNOWLEDGMENTS

This research has been partially supported by the National Institutes of Health through grants from the National Institute on Aging (P01 AG10485) and from the National Institute of General Medical

Services (GM R0l-NS30616-01A2). The important contributions of Drs. Laszlo Prokai, W.M. Wu, Hassan Farag, J.S. Sastry, and J. Simpkins are kindly acknowledged. A significant portion of the work on the TRH analog was performed by X. Ouyang who also has received a Graduate Research Assistantship funded by Genentec Corporation.

REFERENCES

Anderson, W.R.; Simpkins, J.W.; Brewster, M.E.; and Bodor, N. Evidence for the reestablishment of copulatory behavior in castrated male rats with a brain-enhanced estradiol-chemical delivery system. *Pharmacol Biochem Behav* 27:265271, 1987.

Atweh, S.; Simon, J.; and Kuhar, M. Utilization of sodium-dependent high affinity choline uptake *in vitro* as a measure of the activity of cholinergic neurons *in vivo*. *Life Sci* 17:1535-1544, 1975.

Baile, C.A.; McLaughlin, C.L.; and Della-Fera, M.A. Role of cholecystyokinin and opioid peptides in control of food intake. *Physiol Rev* 66:172-234, 1986.

Bassiri, R., and Utiger, R. Metabolism and excretion of exogenous thyrotropin-releasing hormone in humans. J *Clin Innvest* 52:1616-1619, 1973.

Begley, D.J. The interaction of some centrally active drugs with the blood-brain barrier and circumventricular organs. *Prog Brain Res* 91:163-169, 1992.

Begley, D.J.; Squires, L.K.; Zlokovic, B.W.; Mitrovic, D.M.; Hughes, C.C.W.; Revest, P.A.; and Greenwood, J. Permeability of the blood-brain barrier to the immunosuppressive cyclic peptide cyclosporin A. *J Neurochem* 55(4):1222-1230, 1990.

Bernton, E.W.; Long, J.B.; and Holaday, J.W. Opioids and neuropeptides: Mechanisms in circulatory shock. *Fed Proc* 44:290-299, 1985.

Bicknell, R.J. Endogenous opioid peptides and hypothalamic neuro-endocrine neurones. *J Endocrinol* 107:437-446, 1985.

Bodor, N. Redox drug delivery systems for targeting drugs to the brain. *Ann N Y Acad Sci* 507:289-306, 1987.

Bodor, N. New methods of drug targeting. In: Sarel, S.; Mechoulam, R.; and Agranat, I., eds. *Trends in Medicinal Chemistry '90.* Oxford, England: Blackwell Scientific Publications, 1992. pp. 35-44.

Bodor, N., and Brewster, M. Problems of delivery of drugs to the brain. *Pharmacol Ther* 19(3):337-386, 1983.

Bodor, N., and Brewster, M.E. Chemical delivery systems, In: Juliano, R. ed. *Handbook of Experimental Pharmacology.* Vol. 100. Berlin: Springer-Verlag, 1991. pp. 231-284.

Bodor, N., and Kaminski, J. Prodrugs and site-specific chemical delivery systems. In: Bailey, D., ed. *Annual Reports in Medicinal Chemistry.* Vol. XXII. Academic Press, 1987. pp. 303-313.

Bodor, N.; Prokai, L.; Wu, W.-M.; Farag, H.; Jonnalagadda, S.; Kawamura, M.; and Simpkins, J. A strategy for delivering peptides into the central nervous system by sequential metabolism. *Science* 257:1698-1700, 1992.

Bodor, N., and Simpkins, J. Redox delivery system for brain-specific, sustained release of dopamine. *Science* 221(4605):65-67, 1983.

Boler, R.; Enzman, F.; Folkers, K.; Bowers, C.; and Schally, A. The identity of chemical and hormonal properties of the thyrotropin releasing hormone and pyroglutamyl-histidyl-proline amide. *Biochem Biophys Res Commun* 37:705-710, 1969.

Bradbury, A.; Finnie, M.; and Smyth, D. Mechanism of C-terminal amide formation by pituitary enzymes. *Nature* 298:686-688, 1982.

Breese, G.; Cott, J.; Cooper, B.; Prange, A.; Lippton, M.; and Plotnikoff, N. Effects of thyrotropin-releasing hormone (TRH) on the actions of phenobarbital and other centrally acting drugs. *J Pharmacol Exp Ther* 193:11-12, 1975.

Brightman, M.W., and Reese, T.S. Junctions between intimately apposed cell membranes in the vertebrate brain. *J Cell Biol* 40(3):648-677, 1969.

Brownlees, J., and Williams, C.H. Peptidases, Peptides, and the mammalian blood-brain barrier. *J Neurochem* 60(3):793-803, 1993.

Brownstein, M.; Palkovits, M.; Saavedra, R.; Bassiri, R.; and Utiger, R. Thyrotropin-releasing hormone in specific nuclei of rat brain. *Science* 185:267-269, 1974.

Bundgaard, H. *Design of Prodrugs.* Amsterdam: Elsevier Science Publishers, 1985.

Cefalu, W.T, and Pardridge, W.M. Restrictive transport of a lipid-soluble peptide (cyclosporin) through the blood-brain barrier. *J Neurochem* 45(6):1954-1956, 1985.

Fischer, W., and Spiess, J. Identification of a mammalian glutaminyl cyclase converting glutaminyl into pyroglutaminyl in pyroglutamyl peptides. *Proc Natl Acad Sci U S A* 84:3628-3632, 1987.

Frederickson, R.C.A. In: Endogenous opioids and related derivatives. Kuhar, M., and Pasternak, G., eds. *Analgesics: Neurochemical, Behavioral and Clinical Perspectives.* New York: Raven, 1984.

Frederickson, R.C.A., and Geary, L.E. Endogenous opioid peptides: Review of physiological, pharmacological and clinical aspects. *Prog Neurobiol* 19:19-69, 1982.

Frenk, H. Pro- and anticonvulsant actions of morphine and the endogenous opioids: Involvement and interactions of multiple opiate and non-opiate systems. *Brain Res Rev* 6:197-210, 1983.

Grossman, A., and Rees, L.H. The neuroendocrinology of opioid peptides. *Br Med Bull* 39:83-88, 1983.

Hersch, L.; Aboukhair, N.; and Watson, S. Immunohistochemical localization of aminopeptidase M in rat brain and periphery: Relationship of enzyme localization and enkephalin metabolism. *Peptides* 8(3):523-532, 1987.

Hill, R.C.; Marbach, P.; and Scherrer, D. Method for determining the CNS penetration of enkephalin analogs in the rats. *Br J Pharmacol* 72:571, 1981.

Hoek, J., and Rydstrom, J. Physiological roles of nicotinamide nucleotide transhydrogenase. *Biochem J* 254(1):1-10, 1988.

Hoffman, P.L.; Walter, R.; and Bulat, M. An enzymatically stable peptide with activity in the central nervous system: Its penetration through the blood-CSF barrier. *Brain Res* 122(1):87-94, 1977.

Holaday, J.W. Cardiovascular effects of endogenous opiate systems. *Annu Rev Pharmacol Toxicol* 23:541-594, 1983.

Horita, A.; Carino, M.; and Lai, H. Analeptic activity produced by TRH microinjection into basal forebrain area in the rat. *FASEB J* 45:795, 1986.

Horita, A.; Carino, M.; and Smith, J. Effects of TRH on the central nervous system of the rabbit. *Pharmacol Biochem Behav* 5:1 l-l 16, 1976.

Horita, A.; Carino, M.; Zabawska, J.; and Lai, H. TRH analog MK-771 reverses neurochemical and learning deficits in medial septal-lesioned rats. *Peptides* 10(1):121-124, 1989.

Ito, F.; Ito, S.; and Shimizu, N. Transmembrane delivery of polypeptide hormones bypassing the intrinsic cell surface receptors: A conjugate of insulin with alpha 2-macroglobulin (alpha 2M) recognizing both insulin and alpha 2M receptors and its biological activity in relation to endocytic pathways. *Mol Cell Endocrinol* 36(3):165-173, 1984.

Izquierdo, I., and Netto, C.A. Roles of ß-endorphin in behavioral regulation. *Ann N Y Acad Sci* 444:162-177, 1985.

Jackson, I. Controversies in TRH biosynthesis and strategies towards the identification of a TRH precursor. *Ann N Y Acad Sci* 553:71-75, 1989.

Johnson, M.W.; Mitch, W.E.; and Wilcox, C.S. The cardiovascular actions of morphine and the endogenous opioid peptides. *Prog Cardiovasc Dis* 27:435-450, 1985.

Kalivas, P., and Horita, A. Thyrotropin-releasing hormone: Neurogenesis of actions in the pentobarbital narcotized rat. *J Pharmacol Exp Ther* 212:203-210, 1980.

Kraemer, G.; Mueller, R.; Breese, G.; Prange, A., Jr.; Lewis, J.; Morrison, H.; and McKinney, W., Jr. Thyrotropin releasing hormone: Antagonism of pentobarbital narcosis in the monkey. *Pharmacol Biochem Behav* 4:709-712, 1976.

Lampe, T.; Norris, J.; Risse, S.; Owens-Williams, E.; and Keenan, T. Therapeutic potential of thyrotropin-releasing hormone and lecithin coadministration in Alzheimer's disease. In: Iqbal, K.; McLachlan, D.R.C.; Winbald, B.; and Wisniewski, H.M. *Alzheimer's Disease: Basic Mechanisms, Diagnosis and Therapeutic Strategies. New* York: John Wiley and Sons, Ltd., 1991. pp. 643.

McKelvy, J.F. Enzymatic degradation of brain peptides. In: Krieger, D.T.; Brownstein, M.J.; and Martin, J.B., eds. *Brain Peptides.* New York: Wiley-Interscience, 1983. pp. 117-133.

Mellow, A.; Sunderland, T.; Cohen, R.; Lawlor, B.; Hill, J.; Newhouse, P.; Cohen, M.; and Murphy, D. Acute effects of high-dose thyrotropin releasing hormone infusions in Alzheimer's disease. *Psychopharmacology* 98(3):403-407, 1989.

Millan, M.J., and Herz, A. The endocrinology of the opioids. *Int Rev Neurobiol* 26:1-83, 1985.

Miyamota, M.; Nagai, Y.; Norumi, S.; Saji, Y.; and Nagawa, Y. TRH and its novel analog (DN-1417): Antipentobarbital action and involvement of cholinergic mechanism. *Pharmacol Biochem Behav* 17:797-806, 1982.

Neuwelt, E., and Rapoport, S. Modification of the blood-brain barrier in the chemotherapy of malignant brain tumors. *Fed Proc* 43(2):214-219, 1984.

Olson, G.A.; Olson, R.D.; and Kastin, A.J. Endogenous opiates. *Peptides* 6:769-791, 1985.

Pardridge, W. Receptor-mediated peptide transport through the blood-brain barrier. *Endocr Rev* 7(3):314-330, 1986.

Pardridge, W.M. Strategies for drug delivery through the blood-brain barrier. In: Borchardt, R., Repta, A.J., and Stella, V.J., eds. *Directed Drug Delivery: A Multidisciplinary Problem.* Clifton, NJ: Humana Press, 1985. pp. 83-96.

Pardridge, W.M. *Peptide Drug Delivery to the Brain.* New York: Raven Press, 1991.

Pardridge, W.M.; Eisenberg, J.; and Yamada, T. Rapid sequestration and degradation of somatostatin analogs by isolated brain microvessels. *J Neurochem* 44:1178-1184, 1985.

Pardridge, W.M., and Mietus, L.J. Enkephalin and blood-brain barrier: Studies of binding and degradation in isolated brain microvessels. *Endocrinology* 109(4):1138-l 143, 1981.

Pardridge, W.M.; Triguero, D.; and Buciak, J.L. Beta-endorphin chimeric peptides: transport through the blood-brain barrier *in vivo* and cleavage of disulfide linkage by brain. *Endocrinology* 126(2):977-984, 1990*a*.

Pardridge, W.M.; Triguero, D.; Yang, J.; and Cancilla, P.A. Comparison of *in vitro* and *in vivo* models of drug transcytosis through the blood-brain barrier. *J Pharmacol Exp Ther* 253(2):884-891, 1990*b*.

Patel, H.M. Liposomes: bags of challenge. *Biochem Soc Trans* 12(2):333-335, 1984.

Plotnikoff, N.P.; Murgo, J.; Miller, G.C.; Cordes, C.N.; and Faith, R.E. Enkephalins: Immunomodulators. *Fed Proc* 44:118-122, 1985.

Pop, E.; Wu, W.; Shek, E.; and Bodor, N. Brain specific chemical delivery systems for ß-lactam antibiotics: Synthesis and properties of some dihydropyridine and dihydroisoquinoline derivatives of benzylpenicillin. *J Med Chem* 32:1774-1781, 1989.

Poplack, D.G.; Blayer, A.W.; and Horowitz, M.E. In: Wood, J.H., ed. *Neurobiology of Cerebrospinal Fluid.* Vol. I. New York: Plenum Press, 1981. pp. 561-578.

Porreca, F., and Burks, T.F. The spinal cord as a site of opioid effects on gastrointestinal transit in the mouse. *J Pharmacol Exp Ther* 227:22-27, 1983.

Porter, C.; Lotti, V.; and DeFelice, M. The effect of TRH and a related tripeptide, L-N-(2-oxopiperidin-6-yl-carbonyl)-L-histidyl-L-thiazolidine-4-carboxamide (MK-771, OHT), on the depressant action of barbiturates and alcohol in mice and rats. *Life Sci* 21:811-820, 1978.

Powell, M.F. Peptide stability in drug development: *In vitro* peptide degradation in plasma and serum. In: Bristol, J.A., ed. *Annual Reports in Medicinal Chemistry.* Vol. XXVIII. San Diego: Academic Press, Inc., 1993. pp. 285-294.

Prokai, L.; Hsu, B.H.; Farag, H.; and Bodor, N. Desorption chemical ionization, thermospray, and fast atom bombardment mass spectrometry of dihydropyridine⇆|pyridinium salt-type redox systems. *Anal Chem* 61:1723-1728, 1989.

Richter, K.; Kawashima, E.; Egger, R.; and Kreil, G. Biosynthesis of thyrotropin releasing hormone in the skin of Xenopus laevis: Partial sequence of the precursor deduced from cloned cDNA. *EMBO J* 3(3):617-621, 1984.

Sandyk, R. The endogenous opioid system in neurological disorders of the basal ganglia. *Life Sci* 37:1655-1663, 1985.

Santori, E., and Schmidt, D. Effect of MK-771, a TRH analog, on pentobarbital-induced alterations of cholinergic parameters in discrete regions of rat-brain. *Reg Pept* 1:69-74, 1980.

Schick, R., and Schudziarra, V. Physiological, pathophysiological and pharmacological aspects of exogenous and endogenous opiates. *Clin Physiol Biochem* 3:43-60, 1985.

Schmauss, C., and Emrich, H.M. Dopamine and the action of opiates: A reevaluation of the dopamine hypothesis of schizophrenia with special consideration of the role of endogenous opioids in the pathogenesis of schizophrenia. *Biol Psychiatry* 20:1211-1231, 1985.

Schmidt, D. Effects of thyrotropin releasing hormone (TRH) on pentobarbital-induced decrease in cholinergic neuronal activity. *Commun Psychopharmacol* 1:469-473, 1977.

Schwartz, J.; Giros, B.; Gros, C.; Llorens-Cartes, C.; Arrang, J.; Garbarg, M.; and Pollard, H. Novel agents affecting enkephalinergic and histaminergic transmissions in brain. Supplement. *Cephalalgia* 7(6):32-35, 1987.

Shashoua, V.E.; Jacob, J.N.; Ridge, R.; Campbell, A.; and Baldessarini, R.J. Gamma-aminobutyric acid esters: 1. Synthesis, brain uptake, and pharmacological studies of aliphatic and steroid esters of gamma-aminobutyric acid. *J Med Chem* 27(5):659-664, 1984.

Szirtes, T.; Kisfaludy, L.; Palosi, E.; and Szporny, L. Synthesis of thyrotropin-releasing hormone analogs: 1. Complete dissociation of central nervous system effects from thyrotropin-releasing activity. *J Med Chem* 27:741-745, 1984.

Tortella, F.C.; Cowan, A.; and Adler, M.W. Comparison of the anticonvulsant effects of opioid peptides and etorphine. *Life Sci* 29:1039-1045, 1981.

Tortella, F.C.; Long, J.B.; and Holaday, J.W. Endogenous opioid systems: Physiological role in the self-limitation of seizures. *Brain Res* 332:174-178, 1985.

Träuble, H. The movement of molecules across lipid membranes: A molecular theory. *J Membr Biol* 4:193-208, 1971.

Wybran, J. Enkephalins and endorphins as modifiers of the immune system: Present and future. *Fed Proc* 44:92-94, 1985.

Yamaguchi, T.; Yamaguchi, M.; and Lajtha, A. Inhibition of dipeptide transport in mouse brain slices. *J Neural Sci* 10(3):323-329, 1970.

Yarbrough, G. On the neuropharmacology of thyrotropin releasing hormone (TRH). *Prog Neurobiol* 12:291-312, 1979.

Yen, S.S.; Quigley, M.E.; Reid, R.L.; Ropert, J.F.; and Cetel, N.S. Neuroendocrinology of opioid peptides and their role in the control of gonadotropin and prolactin secretion. *Am J Obstet Gynecol* 152:485-493, 1985.

Yoshimoto, T.; Fischl, M.; Orlowski, R.C.; and Walter, R. Post-proline cleaving enzyme and post-proline dipeptidyl aminopeptidase. Comparison of two peptidases with high specificity for proline residues. *J Biol Chem* 253(10):3708-3716, 1978.

Zlokovic, B.; Begley, D.; Segal, M.; Dawson, H.; Rakic, L.; Lipovac, M.; and Mitrovic, D.M. In: Rakic, L.; Begley, D.J.; Davson, H.; and Zlokovic, B., eds. *Peptide and Amino Acid Transport Mechanisms in the Central Nervous System.* London: MacMillan Press, 1988. pp. 3-19.

Zlokovic, B.; Makic, B.; Djuricic, B.; and Davson, H. Kinetic analysis of leucine-enkephalin cellular uptake at the luminal side of the blood-brain barrier of an in situ perfused guinea-pig brain. *J Neurochem* 53(5):1333-1340, 1989.

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Action of Opioid Drugs on the Brain-Reward System

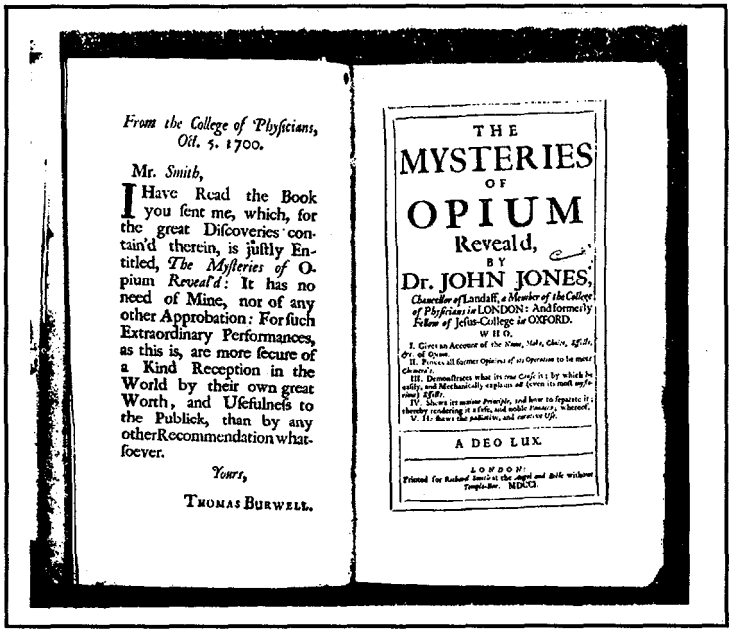
Conan Kornetsky

INTRODUCTION

Mysteries of Opium Revealed, published in the first part of the 18th century, is a title that researchers still could use today, with the possible change of the word "opium" to "opioids." This monograph will reveal much more than *Mysteries;* however, as the chapters in this monograph attest, there is much that has not been revealed. Accompanying the title page of the book is a foreword written by Thomas Burwell of the College of Physicians and Surgeons extolling the virtues of the book (figure 1).

Although the author believes that no compounds have been found that match the opioids in their ability to relieve pain, their nonmedical use is not without consequences. This enigma of the nonmedical use of opioids is stated in the first sentence of *The Road to* H by Isadore Chein and colleagues (1964): "'H' is for heaven; 'H' is for hell; 'H' is for heroin." With some generalization from heroin to other abused opioids, the present chapter deals with the first and third clauses from the first sentence in the Chein and colleagues (1964) book.

The "heaven" alluded to by Chein and colleagues (1964) can be translated into the high or euphoria that has been reported by opiate users. Despite these rewarding effects of opioid drugs, it probably has been only within the past 20 years that extensive research has been directed toward this aspect of drug use. Whatever the reason was for the first use of heroin (e.g., peer pressure, seeking a high), it generally was accepted that continued use was the result of only the avoidance of withdrawal or an adaptive process in which the drug alleviates a pathological condition (Jaffe 1992). As Jaffe (1992) points out, research on the mechanism of the rewarding effects of drugs has rekindled the question, "Do people keep taking drugs primarily to alleviate withdrawal, or do they take them because they continue to experience some of the initial reinforcing effects?" (p. 7). The concept that the hedonic aspect of the drug experience, including the opiates, is a major driving force for drug use has been espoused by a number of investigators (e.g., Jaffe 1992; Koob et al. 1987; Kornetsky et al. 1979; Wise 1980).



From the College of Physicians, Oct. 5. 1700.

Mr. *Smith*,

I Have Read the Book you sent me, which, for the great Discoveries contain'd therein, is justly Entitled, *The Mysteries of Opium Reveal'd*: It has no need of Mine, nor of any other Approbation: For such Extraordinary Performances, as this is, are more secure of a Kind Reception in the World by their own great Worth, and Usefulness to the Publick, than by any other Recommendation whatsoever.

Yours,

THOMAS BURWELL.

THE
MYSTERIES
OF
OPIUM
Reveal'd,
BY
Dr. JOHN JONES,
Chancellor of Landaff, a Member of the College of Physicians in LONDON: And formerly *Fellow of* Jesus-College *in* OXFORD.
WHO.
I. Gives an Account of the Name, Nature, Choice, Specific, &c. of Opium.
II. Proves all former Opinions of its Operation to be most Chimerical.
III. Demonstrates what its true Cause is; by which he easily, and Mechanically explains all (even its most surprising) Effects.
IV. Shews its manner of entring, and how to separate it; thereby rendering it a safe, and noble Remedy, whereof.
V. He knows the palliative, and curative Use.

A DEO LUX.

LONDON:
Printed for Richard Smith at the Angel and Bible without Temple-Bar. MDCI.

FIGURE 1. *Title page and foreword from book by John Jones,* The Mysteries of Opium Revealed, *published in 1701*

Koob (1992) and Koob and Bloom (1988), while not discounting the hedonic aspects of drug-taking behavior, proposed a hypothesis that takes into account the aversive nature of the effect of continued opioid use that manifests itself in the symptoms of withdrawal. Koob (1992) argues that the "same neural elements responsible for 'euphoria' may also be changed in the course of dependence to be responsible for the 'dysphoria' associated with drug removal."

The complexity of the search for pharmacotherapy of drug abuse was made clear by Wikler (1980). He argued convincingly that "relapse is not simply a re-enactment of initial opioid use, but is a 'disease, *sui generis,*' a disease of its own kind" (p. vii). Wikler (1980) points out that therapy must be directed to this separate disease in addition to its initial prevention. In recent years, such approaches (O'Brien et al. 1992) have been designed to extinguish the craving that may be present in the detoxified user.

Despite the myriad of reasons for the nonmedical use of opioids, opioids would not be used nonmedically if they did not cause some pleasurable

effect. What is this effect, and how can the mechanisms involved be understood? There are a number of animal models that have been employed that allow for the study of the rewarding effects of drugs: drug self-administration (the most homologous model), conditioned place preference, drug discrimination, and brain-stimulation reward (BSR). Although each model has advantages as well as disadvantages, it is the latter of these models that will be addressed in this chapter.

EFFECTS OF OPIOID AGONISTS ON BRAIN-STIMULATION REWARD

BSR often is referred to as "intracranial self-stimulation." The demonstration that animals will work in order to receive electrical stimulation to discrete brain areas was first reported by Olds and Milner (1954). Many brain areas will support BSR (Phillips and Fibiger 1989); however, animals with the most robust responding and those easiest to train have stimulating electrodes in the medial forebrain bundle (MFB) or the ventral tegmental area.

The first report of the effects of abused substances on BSR was in Killam and colleagues (1957). The first paper describing the effects of an opioid was by Olds and Travis (1961). In addition to morphine, Olds and Travis determined the effects of chlorpromazine, meprobamate, and pentobarbital. They compared the effects of these drugs on rewarding MFB stimulation to their effect on aversive stimulation to the dorsomedial tegmentum and the medial lemniscus. Except possibly for pentobarbital, there was very little evidence in their experiment that BSR or escape behavior was facilitated by any of these drugs. Since these effects were not different from those caused by other drugs on food-reinforced behavior, the use of BSR for the study of the rewarding effects of opioids was in limbo for over a decade.

Although there were a number of papers on the effects of psychomotor stimulants on BSR during the 1960s (e.g., Stein 1964), the second paper in the literature describing the effects of an opioid did not appear until 1973 (Lorens and Mitchell 1973). They reported that morphine increases rate of response for BSR, but this was not evident until 3 hours after the drug was administered.

Figure 2 summarizes the results from their experiment. Figure 2(a) shows the mean effect of the first dose of morphine given to the rats.

Seen is an immediate decrease in rate of response for the first few hours after the morphine was administered, followed by an increase in response rate. After a few days of spaced morphine administration, the time-effect curve shows very little decrease in rate of response, and the increase in rate of response reached a significant level at approximately 2 hours after morphine administration. This is shown in figure 2(b). This lack of immediate facilitation in BSR is the most common effect seen the first time an animal is administered morphine if rate of response is the dependent variable. Using a rate-independent threshold method of determining the effects of morphine, a lowering of the threshold (increase in sensitivity) the first time an animal is given morphine has been obtained consistently. This is illustrated in figure 3.

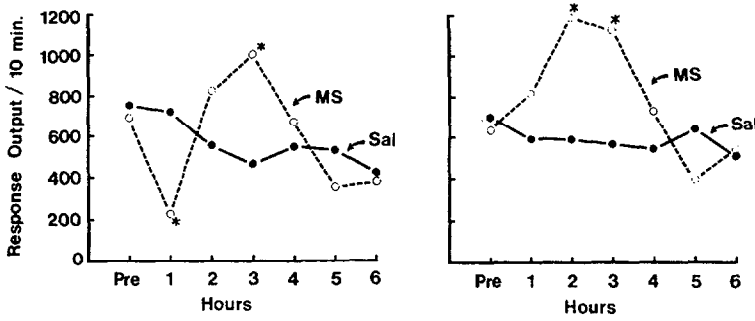


FIGURE 2. *The mean BSR response rate as a function of time after 5 mg/kg of morphine. Panel a shows the effect of the first dose of morphine and panel b the effect after a few spaced doses.*

SOURCE: Drawn from data in Lorens and Mitchell (1974)

Nelsen (1970) compared the effects of morphine sulfate on the amplitude and frequency of the electroencephalograph (EEG) simultaneously recorded from the MFB at the level of the lateral hypothalamus and the mesencephalic reticular formation (MRF). If electrically stimulated, these two sites are rewarding and aversive, respectively. Amplitude of the EEG recorded from the MFB was decreased significantly and increased simultaneously from the MRF. Although changes in EEG frequency did not reach a statistically significant level, frequency of the EEG recorded from the MFB tended to increase while that from the MRF tended to decrease. “Increase frequency/decrease amplitude” in EEG

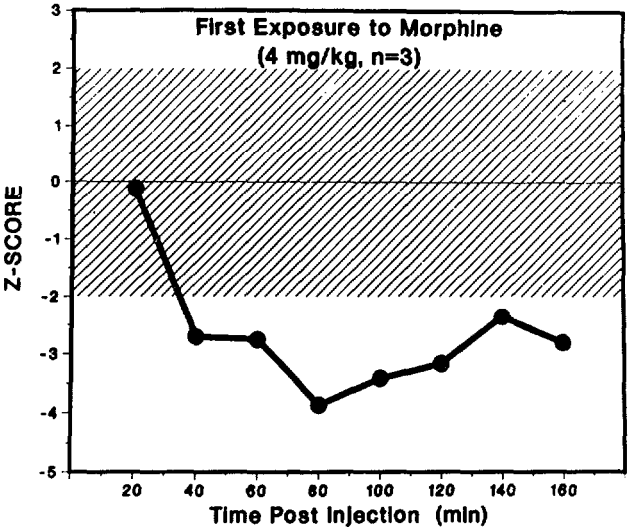


FIGURE 3. *The effect of 4 mg/kg of morphine on the threshold for BSR. A 0 z-score indicates saline levels, and the cross-hatched area indicates the 95 percent confidence limits.*

SOURCE: Izenwasser and Kornetsky (1987)

parlance indicates an activation, with the opposite indicating a decrease in activation. This increase activation in a reward pathway and decrease in a pain pathway after the administration of morphine fits the expected facilitation of reward and depression of pain.

The functional expression of the findings of Nelsen were obtained by Marcus and Kometsky (1974). This experiment demonstrated that morphine lowered the threshold for BSR obtained by stimulation to the MFB and raised the threshold for nociceptive stimulation to the MRF. Subsequently, a number of authors demonstrated that opioid drugs will facilitate responding for BSR, and the chapter author consistently has found that they will lower the reward threshold. Among the earliest papers were Broekkamp et al. (1976), Koob et al. (1975), Lorens (1976) (see review by Esposito and Kometsky 1977; Unterwald and Kometsky 1992; Wauquier et al. 1974). The specificity of the effects of opioids on BSR was demonstrated in Koob and colleagues (1975); they found that heroin increased rate of response for BSR at doses that had no effect on the lever-pressing rate for either food or water reinforcement.

PSYCHOPHYSICAL METHOD FOR DETERMINING BSR THRESHOLD AND EFFECTS OF MORPHINE

The method used for measuring threshold changes is independent of the rate of response and the possible confounding of changes in rate with possible motor effects. A wheel manipulandum was used rather than a lever. The wheel manipulandum allowed for a more direct comparison of the effects of opioids on BSR, with their effects on escape from either foot shock or aversive intracerebral stimulation. Using a wheel manipulandum, rats can be trained to escape from foot shock stimulation in a single brief session. Figure 4 shows a cartoon of the experimental chamber as well as two flow diagrams of the procedure used.

As indicated by flow diagram I, a noncontingent stimulus is presented (S 1) and, if the rat does not respond within a 7.5-second available response time, the trial is terminated, and there is a variable 15-second timeout before the start of the next trial. Flow diagram II illustrates a trial in which the animal turned the wheel manipulandum within the 7.5-second available response time and received a stimulation (S2) of equal intensity to the noncontingent (S 1) stimulation. The intensity of the S1 and, if the animal responds, the accompanying S2, is varied according to the psychophysical method of limits. Figure 5 gives data from an individual animal. Figure 5(a) shows the percent of times the animal responded at each intensity.

The chart in figure 5(b) shows a probit transformation of the percent of responses at each intensity. Only if the slope of the probit-intensity function is significantly greater than 0 can it be argued that the animal is under stimulus control. The obtained threshold after drug treatment for each animal can be transformed to a z-score based on the mean saline treatment threshold (±standard deviation) for that animal. The advantageof such a z-score transformation over percent change from saline is that it takes into account the variability of the saline score and, for the individual animal, a probability statement concerning the extent of effect can be made for a single treatment. Z-scores can be treated like any nominal data (e.g., means, *t*-tests). Figure 3 is an example of such a z-score transformation for a time-effect analysis of 4 mg/kg of morphine, and figure 6 is an example of a dose-effect curve for morphine using the z-score transformation.

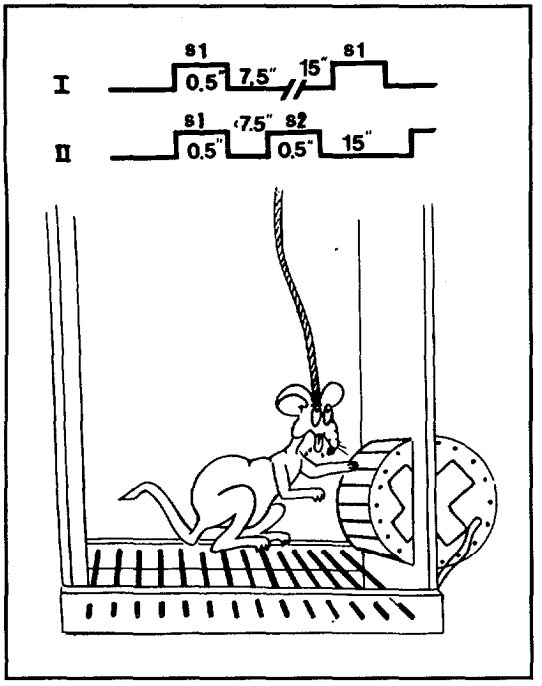


FIGURE 4. *A cartoon of the experimental chamber with rat and stimulating electrode attached. The schematic indicates the sequence of a single trial (see text for explanation).*

HEROIN (DIACETYLMORPHINE)

Heroin and its metabolite, 6-acetylmorphine, lower the BSR threshold. Figure 7, from Hubner and Kornetsky (1992), shows the mean effects of heroin and its two metabolites, 6-acetylmorphine and morphine, on the threshold for rewarding stimulation (figures 7[a], 7[c], and 7[e]) and escape (figures 7[b], 7[d], and 7[fJ) from intracerebral electrical stimulation. The latter procedure is a pain model that was used to determine analgesic effects of opioids (Marcus and Kornetsky 1974; Sasson and Kornetsky 1986).

In the former, the electrodes were in the MFB and, in the latter, they were in the MRF. What is interesting about these data is a comparison of the

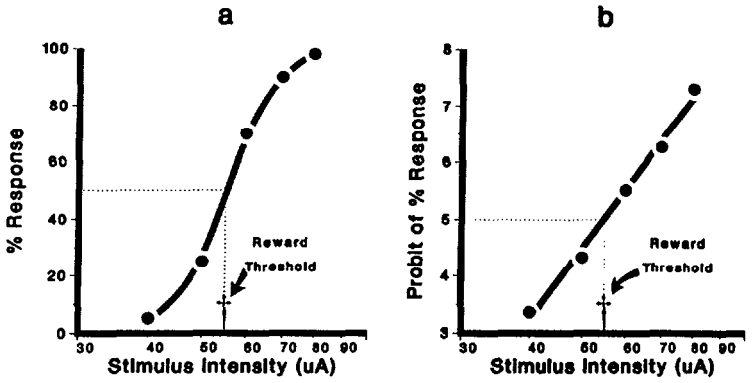


FIGURE 5. *(Panel a) Percent of trials that the animal responded at each intensity. The threshold is indicated by the dagger. (Panel b) Data are converted to a probit of percent responses.*

relative potency of heroin and 6-acetylmorphine to that of morphine on the two procedures. This relationship is more easily seen in table 1.

As shown in table 1, the mean minimum effective doses of heroin and 6-acetylmorphine on BSR are approximately the same, .06 and .08 mg/kg, 33 and 25 times, respectively, more potent than morphine. On the escape procedure, however, heroin and 6-acetylmorphine were only 5 and 3 times, respectively, more potent than morphine. This suggests that heroin or 6-acetylmorphine will affect the brain-reward system at doses well below those that are analgesic.

PHARMACOTHERAPY WITH OPIOID RECEPTOR AGONISTS

The main approaches to pharmacotherapy is the use of drugs that may substitute for the abused opioid and the use of opioid receptor antagonists. The drugs that substitute need not have affinity for opiate receptors; however, they should not have abuse liability by themselves. At one time, one of the most well-known examples of the treatment of opiate addiction with a nonopioid receptor drug was the treatment of opiate use with cocaine by Freud (1974). Unfortunately, it was not believed that cocaine had abuse liability.

A drug that is an opioid receptor agonist and has been used extensively in treating heroin abuse is methadone. Although methadone has been found

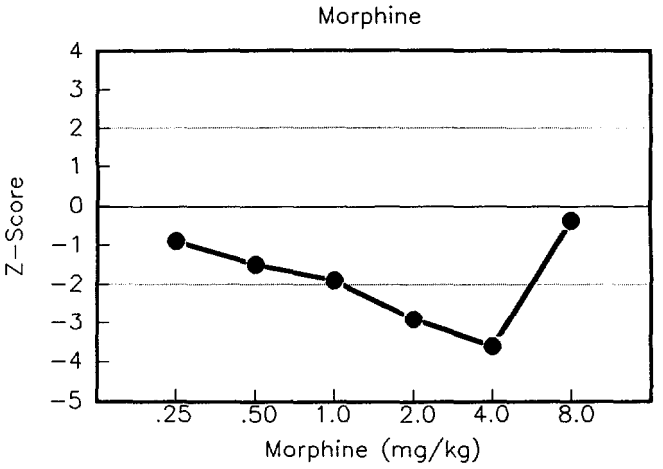


FIGURE 6. *Mean morphine dose-effect curve. Note the inverted U shape. This commonly is seen if sufficiently high doses are included in the curve.*

to lower the threshold for BSR, there are large differences depending A drug that is an opioid receptor agonist and has been used extensively in treating heroin abuse is methadone. Although methadone has been found

TABLE 1. *Relative potencies of heroin, morphine, and 6-acetylmorphine on BSR and escape as a function of minimal effective dose (mg/kg)*

	H	M S	H / M S	6-AMS	6-AMS/MS
Reward	.06	2.0	1l/33	.08	1/25
Escape	.75	4.0	1/5	1.25	1/3

KEY: H = heroin; MS = morphine; 6-AMS = 6-acetylmorphine

SOURCE: Taken from data in Hubner and Kornetsky (1992)

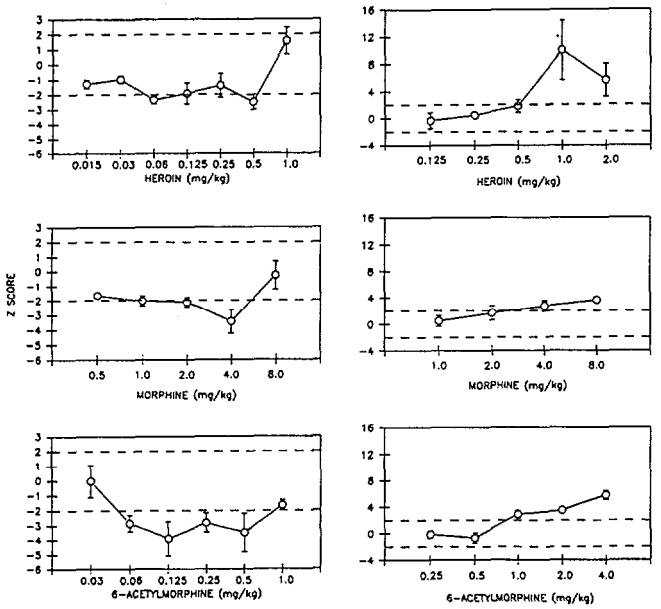


FIGURE 7. *(Panels a, c, and e) Dose-effect curve for the effects* of *heroin, morphine, and 6-acetylmorphine on the BSR threshold. (Panels b, d, and f) Dose-eflect curves for the effects* of *the same drugs on the threshold for escape from aversive electrical stimulation delivered to the mesencephalic reticular formation.*

SOURCE: Hubner and Kornetsky (1992)

upon the history of the animals. Figure 8 shows the mean effect of methadone in six animals. Although the effect appears markedly robust, it is accounted for mainly by four of the animals; these four animals had extensive experience with cocaine prior to the methadone experiment. Methadone had only minimal effects in lowering the threshold in the two animals with no previous cocaine experience.

Further confounding of this result is that the four animals that previously received cocaine also had been administered repeated doses of the opiate antagonist nalmefene. It is possible that cocaine sensitizes the animal to methadone, that repeated nalmefene treatment causes some long-lasting up-regulation of the opiate receptor that results in an increased effect of

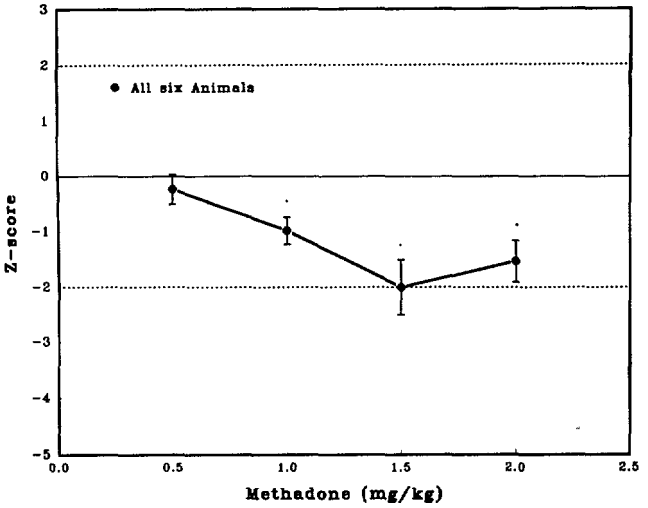


FIGURE 8. *Mean effects of methadone on BSR threshold*

methadone on BSR, or both. At the present time, this difference has not been resolved.

Buphrenorphine, a mixed-agonist antagonist, decreases heroin self-administration in humans (Mello et al. 1982). The question is whether it blocks the euphoria of the abused substance or merely substitutes one effect for another. Buphrenorphine, by itself, has been found to lower the BSR threshold significantly and robustly (Hubner and Kornetsky 1988). This suggests that, to the extent that BSR is a model for drug-induced euphoria, its use in the pharmacotherapy of heroin abuse primarily would be that of substitution.

OPIOID RECEPTOR ANTAGONIST

The two recent reviews of the effects of opioid antagonists on BSR were by Shaefer (1988) and Trujillo and colleagues (1989). Both reviews point out that there is a great deal of inconsistency in the effects of the antagonists. Trujillo and colleagues (1989) argue that differences in the findings are due to electrode placement, the length of the test sessions, or both. However, in their review, there are experiments in which positive results have been obtained from so-called negative sites (hypothalamic or MFB electrode placements) and experiments using short test periods.

The opposite also is found: Negative results have been obtained from so-called sensitive placement sites and testing for longer durations. Shaefer (1988), in his review, attributed the differences between investigations to reinforcement schedule effects.

In repeated experiments in which the effects of naloxone on the BSR threshold have been studied, no evidence has been found of threshold-raising or lowering effects (Bain and Kornetsky 1987; Esposito et al. 1980; Huston-Lyons and Kornetsky 1992; Knapp and Kometsky 1989). Similarly, it has been found that the opioid receptor antagonist nalmefene has no effect on the BSR threshold (Kometsky, unpublished data). Also, in one experiment in which naloxone was administered daily for 5 days (Perry et al. 1981), no effect was observed. Although an opioid antagonist may decrease rate of response, the Perry and colleagues (1981) experiment, using a rate-independent method of BSR, suggests that a tonic activation of an endorphinergic system is not necessary for the intracranial stimulation to be rewarding. If this is the case, then the threshold-lowering effects of opioids on BSR and their reinforcing effects are the result of the opioid modulating some other system.

DOPAMINE AGONISTS AND ANTAGONISTS

If the reinforcing effects of opioids involve the modulation of some other neurotransmitter system or systems, then there should be evidence of behavioral interaction with the system. Substance abusers often will self-administer opioids in combination with other drugs. Among the common combinations are heroin plus cocaine, "speedball," or d-amphetamine. Both of these psychomotor stimulants, cocaine (Kometsky, unpublished data) and d-amphetamine (Hubner et al. 1987), will potentiate the threshold-lowering effects of morphine on BSR. To test the relative contribution of action on the dopamine and noradrenergic systems to this synergistic effect, Izenwasser and Kometsky (1989) determined the interaction of morphine with more specific dopamine and noradrenergic agonists. They combined morphine with both amfonelic acid (AFA), a more specific dopamine agonist than amphetamine or cocaine, and the relatively specific adrenergic reuptake blocker nisoxetine. Nisoxetine had no significant effect by itself on BSR and did not potentiate the effect of morphine. The opposite result was obtained with AFA. This drug not only lowered the threshold by itself, but also significantly potentiated the effects of morphine. These findings implicate dopamine but not norepinephrine in the interaction of opioids with psychomotor stimulants

and support the hypothesis that dopamine may be critical for the reinforcing effects of the abused opioids. It should be pointed out that this synergistic effect on BSR by AFA and not by nisoxetine is not seen when morphine is combined with these two compounds on a model used for studying the analgesic effect of opioids (Izenwasser and Kometsky 1988). AFA blocks the analgesic action of morphine, while nisoxetine potentiates the analgesic action of morphine. These results suggest that the potentiation of morphine analgesia in human patients by d-amphetamine (Forrest et al. 1977) primarily is a result of d-amphetamine's noradrenergic activity.

Because of the interaction seen between dopamine and opioid agonists, it would be reasonable to consider dopamine antagonists as drugs that might be useful in the pharmacotherapy of opioid abuse. The major problem is that, unlike the opiate antagonists, the dopamine antagonists are not without major intrinsic activity. Among these effects is that they raise the threshold for BSR; haloperidol (Esposito et al. 1979), chlorpromazine (Esposito et al. 1981), and pimozide (Bird and Kometsky 1990) all raise the BSR threshold. Despite the fact that the neuroleptic drugs do alter the BSR threshold, it has been found that doses of dopamine antagonists that have no significant effects by themselves will block the threshold-lowering effect of opioids on BSR (Sarkar et al. 1992). Figure 9 illustrates this blocking-of-morphine effect by the D2 antagonist pimozide. The D1-D2 antagonist cis-flupenthixol will block the threshold-lowering effects of the mu agonist Tyr-D.Ala-Gly-(Me)Phe-Gly.ol (DAMGO) (Duvauchelle et al. 1994).

Among the experiments of opioid-dopamine interaction, the chlorpromazine experiment (Esposito et al. 1981), in particular, suggests an interesting combination that may be useful in treating opioid abuse. Because, in previous experiments, naloxone was found to block the threshold-lowering effects of drugs with dopamine agonist activity, it was postulated that naloxone should potentiate the threshold-raising effects of chlorpromazine on BSR. Figure 10 shows that naloxone, in fact, did potentiate the threshold-raising effects of chlorpromazine. The addition of an opioid antagonist to the dopamine antagonist might allow for lower doses of the latter, thus avoiding some of the side effects of the dopamine antagonists. Because of the lack of specificity of chlorpromazine on neurotransmitter systems, the effect of this combination of dopamine and opioid antagonist needs to be replicated using a more specific dopamine antagonist.

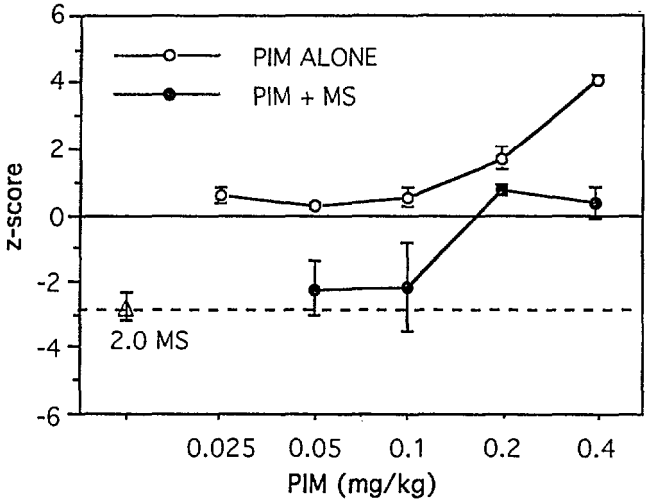


FIGURE 9. *Effects of pimozide alone and in combination with 2 mg/kg of morphine on the BSR threshold*

HISTAMINE

Other possible candidates that this work suggests are seen in experiments suggesting a role for histamine in its interaction with pentazocine. A combination of drugs that was popular in the 1980s was pentazocine and the antihistamine tripelennamine, commonly called "T's and Blues." Tripelennamine and pentazocine have been found to have synergistic effects in lowering the BSR threshold (Unterwald and Kornetsky 1984). Because tripelennamine has affinity for receptors other than histamine, a more direct experiment would be to determine if L-histidine, a drug that raises brain histamine levels, would block the effects of pentazocine on BSR. Figure 11 illustrates a threshold-lowering dose of pentazocine alone and in combination with various doses of L-histidine (Rassnick and Kometsky 1991). L-histidine by itself had no significant effect on the BSR threshold. However, it significantly attenuated the threshold-lowering effect of pentazocine.

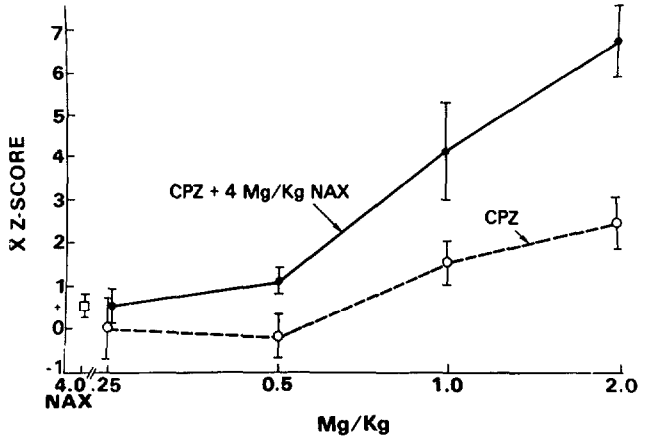


FIGURE 10. *Effects of chlorpromazine alone and in combination with 4 mg/kg of naloxone on the BSR threshold*

SOURCE: Esposito and colleagues (1981)

BSR AS A MODEL FOR SCREENING FOR PHARMACOTHERAPEUTIC DRUGS

A large number of experiments from a number of laboratories (e.g., Broekkamp et al. 1976; Marcus and Kometsky 1974; Stein 1964; Unterwald and Kometsky 1992; Wise 1980), using a variety of methods for determining the threshold for rewarding brain stimulation, have established that most abused substances increase the sensitivity of animals to such stimulation. There is considerable evidence that a major substrate for this increase in sensitivity is related to the action of the abuse substances, either directly or indirectly, on the ascending mesolimbic dopamine system (Phillips and Fibiger 1989; Unterwald and Kometsky 1992; Wise 1980). It also is reasonably clear that dopamine probably does not play exactly the same role in mediating the rewarding effects of all abused substances. Of the major models used to predict abuse liability, BSR (although not as homologous a model of drug abuse as self-administration) has a number of advantages. If proper methods are employed, the effects of opioids and putative therapeutic drugs on the BSR threshold can be measured independent of any motor effects. Also, these effects can be determined reliably and validly with as few as four to six animals.

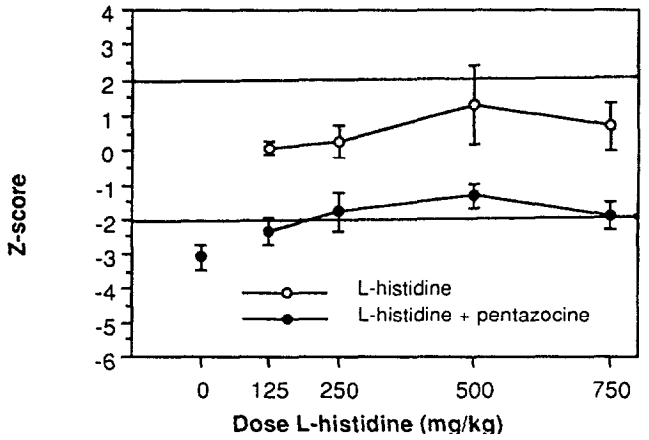


FIGURE 11. *Effects of L-histidine alone and in combination with pentazocine (dose that significantly lowers the threshold in each individual animal) on the BSR threshold*

A major problem is not the finding of adequate behavioral models but defining what the effect of the ideal therapeutic drug should do. There already are good opioid antagonists and drugs that will substitute for heroin. A drug that substitutes ideally should have only mild abuse liability. This latter treatment might be called *benign substitution.* If the receptor or receptors specifically involved in the rewarding effects of would be useful for all abusers of opioids. Wikler (1980) in his use of the opioids could be found, a drug more useful than those currently in hand might be found. If such a drug were to be found, it is unlikely that it term sui generis "a disease of its own kind," was referring to the physically dependent individual; however, generalizing from this, it is clear that the continued use of opiates has many etiological reasons, and each may be a sui generis.

REFERENCES

Bain, G.T., and Kometsky, C. Naloxone attenuation of the effects of cocaine on rewarding brain stimulation. *Life Sci* 40:1119-1 125, 1987.

Bird, M., and Kometsky, C. Dissociation of the attentional and motivational effects of pimozide on the threshold for rewarding brain stimulation. *Neuropsychopharmacology* 3:33-40, 1990.

Broekkamp, C.L.; Van den Bogaard, J.J.; Heijnen, H.J.; Rops, R.H.; Cools, A.R.; and Van Rossum, J.M. Separation of inhibiting and stimulating effects of morphine on self-stimulation behaviour by intracerebral microinjections. *Eur J Pharmacol* 36:443-446, 1976.

Chein, I.; Gerard, D.L.; Lee, R.S.; and Rosenfeld, E. *The Road to H.* New York: Basic Books, Inc., 1964.

Duvauchelle, C.L.; Fleming, S.M.; and Kometsky, C. Micro-injections of DAMGO into the nucleus accumbens and olfactory tubercle lower brain stimulation reward thresholds. In: Harris, L.S., ed. *Problems of Drug Dependence, 1993: Proceedings of the 55th Annual Scientific Meeting, The College on Problems of Drug Dependence, Inc.* Vol. II, *Abstracts*. National Institute on Drug Abuse Research Monograph 144. NIH Pub No. 94-3749. Washington, DC: Supt. of Docs., U.S. Govt. Print. Off., 1994. p. 91.

Esposito, R.U.; Faulkner, W.; and Kometsky, C. Specific modulation of brain stimulation reward by haloperidol. *Pharmacol Biochem Behav* 10:937-940, 1979.

Esposito, R.U., and Kometsky, C. Morphine lowering of self-stimulation thresholds: Lack of tolerance with long-term administration. *Science* 195:189-191, 1977.

Esposito, R.U.; Perry, W.; and Kometsky, C. Effects of d-amphetamine and naloxone on brain stimulation reward. *Psychopharmacology* 68:187-191, 1980.

Esposito, R.U.; Perry, W.; and Kometsky, C. Chlorpromazine and brain-stimulation reward: Potentiation of effects by naloxone. *Pharmacol Biochem Behav* 15:903-905, 1981.

Forrest, W.H.; Brown, B.W.; Brown, C.R.; Defalque, R.; Gold, M.; Gordon, H.E.; James, K.E.; Katz, J.; Mahler, D.L.; Schroff, P.; and Teutsch, G. Dextroamphetamine with morphine for the treatment of postoperative pain. *N Engl J Med* 296:712-715, 1977.

Freud, S. *Cocaine Papers.* (Byck, R., ed.) Ed. compil. New York: New American Library, 1974.

Hubner, C.B.; Bain, G.T.; and Kometsky, C. The combined effects of morphine and d-amphetamine on the threshold for brain stimulation reward. *Pharmacol Biochem Behav* 28:311-315, 1987.

Hubner, C.B., and Kometsky, C. The reinforcing properties of the mixed agonist-antagonist buprenorphine as assessed by brain-stimulation reward. *Pharmacol Biochem Behav* 30:195-197, 1988.

Hubner, C.B., and Kometsky, C. Heroin, 6-acetylmorphine and morphine effects on threshold for rewarding and aversive brain stimulation. *J Pharmacol Exp Ther* 260:1562-567, 1992.

Huston-Lyons, D., and Kometsky, C. Effects of nicotine on the threshold for rewarding brain stimulation in rats. *Pharmacol Biochem Behav* 41:755-759, 1992.

Izenwasser, S.E., and Kometsky, C. Pharmacological effects of morphine on brain-stimulation reward. *Psychopharmacology* 93:136-137, 1987.

Izenwasser, S., and Kometsky, C. Potentiation of morphine analgesia by d-amphetamine is mediated by norepinephrine and not dopamine. *Pain* 33:363-368, 1988.

Izenwasser, S., and Kometsky, C. The effect of amfonelic acid or nisoxetine in combination with morphine on brain-stimulation reward. *Pharmacol Biochem Behav* 32:983-986, 1989.

Jaffe, J.H. Current concepts of addiction. In: O'Brien, C.P., and Jaffe, J.H., eds. *Addictive States.* Vol. 70. New York: Raven Press, 1992. pp. 1-21.

Killam, K.F.; Olds, J.; and Sinclair, J. Further studies on the effects of centrally acting drugs on self-stimulation. *J Pharmacol Exp Ther* 119:157, 1957.

Knapp, C., and Kometsky, C. The effects of amfonelic acid alone and in combination with naloxone on brain-stimulation reward. *Pharmacol Biochem Behav* 32:977-982, 1989.

Koob, G.F. Neurobiological mechanisms in cocaine and opiate dependence. In: O'Brien, C.P., and Jaffe, J.H., eds. *Addictive States.* Vol. 70. New York: Raven Press, 1992. pp. 79-92.

Koob, G.F., and Bloom, F.E. Cellular and molecular mechanisms of drug dependence. *Science* 242:715-723, 1988.

Koob, G.F.; Spector, N.H.; and Meyerhoff, J.L. Effects of heroin on lever pressing for intracranial self-stimulation, food and water in the rat. *Psychopharmacologia* 42:231-234, 1975.

Koob, G.F.; Vaccarino, F.; Amalaic, M.; and Bloom, F.E. Positive reinforcement properties of drugs: search for neural substrates. In: Engel, J., and Orland, L., eds. *Brain Reward Systems and Abuse.* New York: Raven Press, 1987. pp. 35-50.

Kometsky, C.; Esposito, R.U.; McLean, S.; and Jacobson, J.O. Intracranial self-stimulation thresholds: A model for the hedonic effects of drugs of abuse. *Arch Gen Psychiatry* 36:289-292, 1979.

Lorens, S.A. Comparison of the effects of morphine on hypothalamic and medial frontal cortex self-stimulation in the rat. *Psychopharmacology* 48:217-224, 1976.

Lorens, S.A., and Mitchell, C.L. Influence of morphine on lateral hypothalamic self-stimulation in the rat. *Psychopharmacologia* 32:271-277, 1973.

Marcus, R., and Kornetsky, C. Negative and positive intracranial reinforcement thresholds: Effects of morphine. *Psychopharmacologia* 38:1-13, 1974.

Mello, N.K.; Mendelson, J.H.; and Kuehnle, J.C. Buprenorphine effects on human heroin self-administration: An operant analysis. *J Pharmacol Exp Ther* 223:30-39, 1982.

Nelsen, J.M. "Single Dose Tolerance to Morphine Sulfate: Electroencephalographic Correlates in Central Motivational Systems." Ph.D. diss., Boston University, 1970.

O'Brien, C.P.; Childress, A.R.; McLellan, A.T.; and Ehrman, R. A learning model of addiction. In: O'Brien, C.P., and Jaffe, J.H., eds. *Addictive States.* Vol. 70. New York: Raven Press, 1992. pp. 157-177.

Olds, J., and Milner, P. Positive reinforcement produced by electrical stimulation of septal area and other regions of rat brain. *J Comp Physiol Psychol* 47:419-427, 1954.

Olds, J., and Travis, R.P. Effects of chlorpromazine, meprobamate, pentobarbital and morphine on self-stimulation. *J Pharmacol Exp Ther* 128:397-404, 196l.

Perry, W.; Esposito, R.U.; and Kometsky, C. Effects of chronic naloxone treatment on brain-stimulation reward. *Pharmacol Biochem Behav* 14:247-249, 1981.

Phillips, A.G., and Fibiger, H.C. Neuroanatomical bases of intracranial self-stimulation: untangling the Gordian knot. In: Liebman, J.M., and Cooper, S.J., eds. *The Neuropharmacological Basis of Reward.* Oxford: Clarendon Press, 1989. pp. 66-105.

Rassnick, S., and Kometsky, C. L-histidine attenuates the effects of pentazocine on rewarding brain-stimulation. *Life Sci* 48:1729-1736, 1991.

Sarkar, M.; Huston-Lyons, D.; and Kometsky, C. Reward-specific blockade of morphine's effect on brain-stimulation reward by pimozide but not SCH23390. *Abstr Soc Neurosci* 18:374, 1992.

Sasson, S., and Kometsky, C. Evidence for a supraspinal analgesic effect with cyclazocine and pentazocine. *Life Sci* 38:21-26, 1986.

Shaefer, G.J. Opiate antagonists and rewarding brain stimulation. *Neurosci Biobehav Rev* 12:1-17, 1988.

Stein, L. Self-stimulation of the brain and the central stimulant action of amphetamine. *Fed Proc* 23:836-850, 1964.

Trujillo, K.A.; Belluzzi, J.D.; and Stein, L. Opiate antagonists and self-stimulation: Extinction-like response patterns suggest selective reward deficit. *Brain Res* 492:15-28, 1989.

Unterwald, E.M., and Kometsky, C. Effects of concomitant pentazocine and tripelennamine on brain-stimulation reward. *Pharmacol Biochem Behav* 21:961-964, 1984.

Unterwald, E.M., and Kometsky, C. Reinforcing effects of opiates-modulation by dopamine. In: Hammer, R., ed. *The Neurobiology of Opiates.* Boca Raton, FL: CRC Press, Inc., 1992. pp. 361-392.

Wauquier, A.; Niemegeers, C.J.E.; and Lal, H. Differential antagonism by naloxone of inhibitory effects of haloperidol and morphine on brain stimulation. *Psychopharmacologia* 37:303-310, 1974.

Wikler, A. *Opioid Dependence.* New York: Plenum Press, 1980.

Wise, R.A. Action of drugs of abuse on brain reward systems. *Pharmacol Biochem Behav* 13:213-223,1980.

ACKNOWLEDGMENTS

This work was supported in part by grants DA02326 and DA05100 and Research Scientist Award DA00099 from the National Institute on Drug Abuse to Dr. Kometsky .

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Drugs That Modify Opioid Tolerance, Physical Dependence, and Abstinence Symptoms: Preclinical and Clinical Studies

Hemendra N. Bhargava

INTRODUCTION

Opioids are used widely for the treatment of pain of moderate to severe intensity, like the pain in carcinoma, biliary or renal colic and postoperative pain. Opioids relieve painful stimuli by interacting with three major types of receptors, namely, μ, δ, and κ, in various brain regions and the spinal cord. The typical agonists for these receptors are morphine or Tyr-D.Ala-Gly-(Me)Phe-Gly.ol enkephalin (DAMGO) (μ), ethylketocyclazocine or U-50,488H(κ), and D-Pen^2, D-Pen^5 enkephalin (DPDPE) (6) (Bhargava 1991*a*). Endogenous opioid peptides also have been identified, isolated, and are known as enkephalins, dynorphins, and ß-endorphin. The enkephalins and dynorphins exhibit affinity predominantly for δ and κ receptors, whereas ß-endorphin has mixed action on μ and δ receptors.

Both exogenous and endogenous opioids produce pain relief; however, chronic administration of these drugs for a long period of time, particularly at high doses, results in the development of tolerance to their analgesic and other pharmacological actions. The development of physical dependence on these drugs, however, depends on the nature of the drug. In general, drugs acting on μ opioid receptors produce a high degree of physical dependence, as evidenced by the appearance of severe distressing physical symptoms called abstinence syndrome. Some but not all of these symptoms are relieved by readministering the same drug or similar type of drugs. These drugs (e.g., morphine or heroin) are considered to be highly addictive. κ opioid drugs do not produce physical dependence and generally are labeled as nonaddicting drugs. 8 opioids, which are all peptides in nature, generally do not produce physical dependence; however, drugs like ß-endorphin, when given chronically into the central nervous system (CNS), can produce addictive behavior. Addiction generally constitutes tolerance, physical dependence, abstinence syndrome, and self-administrative behavior.

The biochemical or molecular mechanisms involved in these four processes still are the subject of intense investigation. A wide variety of agents have been reported to modify the opioid addiction processes not only in animals but also in humans. The purpose of this review is to identify these agents, classify them in various categories based on their chemical characteristics or specific receptor interaction, analyze their actions as reported by different investigators, and identify consistencies or inconsistencies in their actions. Before the effects of various drugs on the addictive processes are described, a brief description of the animal models used for developing tolerance, physical dependence, and self-administration will be provided. Major emphasis will be given to morphine, which produces tolerance, dependence, abstinence syndrome, and self-administrative behavior.

PROCEDURES FOR THE INDUCTION OF OPIOID TOLERANCE AND PHYSICAL DEPENDENCE

Experimental animals as well as humans have been utilized to determine the effects of drugs on opioid addiction processes. The animals include mice, rats, guinea pigs, and monkeys. Morphine produces a plethora of pharmacological actions. Chronic administration of morphine produces tolerance to its analgesic, hypothermic, respiratory-depressant, euphoric, cataleptic, locomotor-depressant, and stimulant actions. The majority of the studies have concentrated on the tolerance to its analgesic action. The analgesic response has been measured by the tail flick test, hot plate test, or the acetic acid-induced writhing test. Chronic administration of morphine also results in the development of physical dependence, as evidenced by the appearance of distressing physical symptoms following its withdrawal. These symptoms and their intensity vary with the degree of dependence and the species used. In the majority of the studies, rodents have been used. For further confirmation of the results, guinea pigs or monkeys have been utilized. The details of the procedures used have been reviewed (Bhargava, in press).

Earlier studies had utilized multiple intraperitoneal (i.p.) or subcutaneous (s.c.) injections of morphine in increasing doses to develop dependence in the rat. Intravenous (i.v.) administration of morphine, as well as food admixed with morphine, has been used for both inducing morphine addiction and studying self-administrative behavior in rats and monkeys. Morphine tolerance/dependence (MTD) also has been induced in the rat by administering the drug in drinking water, saline, or sucrose solution

and in mice by the s.c. implantation of specifically formulated pellets of morphine freebase. Although the pellet implantation procedure offers convenience, it suffers from the disadvantage that there is a rapid release followed by slow sustained release of morphine from the pellet. The situation may be slightly different from that observed in humans. Two other methods have been used. They include injection of a large dose (100 mg/kg) of morphine in mice to induce a state of acute tolerance 4 hours afterward and implantation of osmotic minipumps containing morphine solution that can be delivered at a predetermined rate. The minipumps can be used to deliver the morphine s.c. or directly into the brain.

As indicated earlier, the symptoms of withdrawal can be induced either by abrupt termination of morphine treatment or by injecting an opioid antagonist like naloxone, nalorphine, naltrexone, or levallorphan. The symptoms of abrupt and antagonist-induced withdrawal differ in their quality, onset, intensity, and duration. In morphine-dependent subjects, the nature of the symptoms depends on the species studied. In the majority of the studies in mice, naloxone- or naltrexone-induced stereotyped jumping behavior (a hyperactivity response), and changes in the body weight and body temperature have been monitored. In rats, several other symptoms like teeth chattering, wet dog shakes, ptosis, penile erection, and lacrimation also have been monitored, in addition to jumping or escape behavior and changes in the body weight and body temperature (Bhargava, in press). Drugs are known to modify only some but not all the symptoms of the abstinence syndrome.

In the following sections, the effects of drugs known to modify the development of opioid tolerance and physical dependence will be described. Additionally, brief statements will be made on the specific mechanism involved that led to the use of specific agents. However, for greater detail, the reader can refer to a recent review (Bhargava, in press).

DRUGS THAT MODIFY OPIOID TOLERANCE AND PHYSICAL DEPENDENCE PROCESSES

Drugs Related to Opioids

Opioid Agonists and Antagonists. Opioid antagonists like nalorphine, naloxone, and naltrexone, which antagonize the responses mediated by μ, δ, and κ agonists modify tolerance and dependence on morphine.

Although the changes in brain and spinal cord μ, δ, and κ opioid receptors are not found to be consistent following the chronic morphine administration and its withdrawal, several selective and nonselective opioid antagonists have been used to reverse the morphine tolerance and physical dependence processes. Mushlin and Cochin (1976) showed that naloxone injected 35 minutes after morphine pretreatment prevented the development of tolerance to morphine in the rat. The inhibition by naloxone of morphine-induced tolerance and dependence in mice was dose and time dependent. Such an effect was observed whether the tolerance was induced by injection of a single large dose of morphine (100 mg/kg, s.c.) or when morphine was injected on a chronic basis using a slow release preparation (Yano and Takemori 1977). A single i.p. injection of naltrexone (20 mg/kg) partially inhibited the development of physical dependence on morphine in mice rendered dependent by implantation of a pellet containing 75 mg of morphine base for 3 days. The effect of naltrexone was much more pronounced when given prior to and during the development of dependence. The effect also was seen when naltrexone was injected 1 day after the pellet implantation (Bhargava 1978*a*). A single pellet of naltrexone containing 10 mg of the drug completely blocked the tolerance to the analgesic and hyperthermic effects of morphine, as well as the development of physical dependence on morphine in the rat. In this study, the tolerance or dependence was induced by s.c. implantation of six morphine pellets (75 mg each) during a 7-day period (Bhargava et al. 1994*a*).

Conflicting reports exist on the involvement of δ and κ opioid receptors in MTD (Bhargava 1991*a*). ß-funaltrexamine, a highly sensitive μ antagonist (Ward et al. 1982) has been shown to inhibit the development of physical dependence on morphine in rats and monkeys (Aceto et al. 1986). The δ receptors antagonists naltrindole and its analog, naltrindole 5′-isothiocyanate, inhibited the tolerance and physical dependence on morphine in mice induced either by injection of a single dose of morphine or by the pellet implantation procedure. These δ antagonists, unlike μ antagonists, did not alter the analgesic response to morphine (Abdelhamid et al. 1991).

The development of tolerance to morphine in the mouse has been shown to be blocked by i.p. or intrathecal (i.t.) injections but not intracerebroventricular (i.c.v.) injections of U-50,488H, a κ opioid agonist. The suppressive action of U-50,488H on morphine tolerance was antagonized by norbinaltorphimine, a κ opioid antagonist, when given i.p. or i.t. but not i.c.v. These studies indicate a role for κ opioid receptors in morphine

tolerance (Takahashi et al. 1991). A similar effect of U-50,488H has been demonstrated in the rat (Yamamoto et al. 1988). However, studies showed that U-50,488H (25 mg/kg) given i.p. twice a day did not block tolerance to the analgesic or hyperthermic effects of morphine in the rat, induced by either implanting four pellets during a 3-day period or six pellets during a 7-day period (Bhargava et al. 1991). These results are in agreement with the studies of Fukagawa and colleagues (1989), who showed that U-50,488H does not affect the development of morphine dependence in the rat. The disparity between the above studies may be due to the method used to induce morphine tolerance.

Opioid Peptides

Enkephalins. Soon after the discovery of opioid receptors in the mammalian brain, two pentapeptides, leucine- and methionine-enkephalin (Leu-enkephalin and Met-enkephalin) were isolated and were found to have morphine-like activity in various assay systems (Bhargava 1977, 19783; Buscher et al. 1976; Hughes et al. 1975). The effects of Leu-enkephalin and Met-enkephalin on tolerance and dependence on morphine were determined in mice. Leu-enkephalin (5 mg/kg) enhanced not only the analgesic potency of morphine but also the development of acute tolerance and dependence to morphine induced by injecting morphine (100 mg/kg, s.c.). Met-enkephalin did not affect morphine analgesia or development of the tolerance and dependence process (Vaught and Takemori 1979). Studies in E.L. Way's laboratory (Chapman et al. 1980) showed that Leu-enkephalin had no effect on morphine analgesia, tolerance, or dependence development in mice. Low doses of Met-enkephahn (i.c.v.) antagonized morphine analgesia without affecting tolerance or dependence development. The reasons for the discrepancy between the studies of Vaught and Takemori (1979) and Chapman and colleagues (1980) are not apparent at this time. However, the studies of Chapman and colleagues (1980) were carried out with peptides obtained from three sources and on several strains of mice.

Dynorphin A(1-13). Dynorphin A(1-13) administered i.v. in doses of 2.5 and 5.0 μmol/kg inhibited the expression of morphine withdrawal and tolerance in mice (Takemori et al. 1992). Mice were implanted with a morphine pellet for 3 days. Dyn A(1-13) was injected 5 minutes prior to injection of naloxone. Dyn A(1-13) raised the naloxone ED, value for the jumping response. The ED, value of morphine for the analgesic response in morphine-tolerant mice also was increased. This increase was reversed in a dose-dependent manner by dyn A(1-13). Although the

mechanism by which dyn A(1-13) modifies morphine effects is not clear, studies have demonstrated that morphine tolerance and abstinence are associated with increases in the levels of dyn A(1-13) in several brain regions but decreases in the spinal cord (Rattan et al. 1992). In particular, a long-lasting depletion in the dyn A(1-13) levels was found in the spinal cord of morphine tolerant or dependent rats. This may partially explain the efficacy of i.t. administration but not of i.c.v. administration of dyn A(1-13) in the modification of the expression of morphine tolerance and withdrawal.

Nonopioid Peptides

Thyrotropin-Releasing Hormone (TRH). TRH, a tripeptide (pGlu-His-Pro-NH&, besides its endocrine activity of releasing thyrotropin and prolactin, also possesses many pharmacological actions in the CNS (Bhargava et al. 1983). Studies have shown that TRH (4-16 mg/kg, s.c.) prevents the development of tolerance to the analgesic but not the hypothermic actions of morphine in the mice and rats, as well as development of physical dependence in mice (Bhargava et al. 1983; Ramarao and Bhargava 1990). It also should be noted that TRH does not affect morphine analgesia nor does it modify the binding of ^{3}H-ligands to opioid receptors in the brain (Bhargava et al. 1983). Therefore, it appears that the inhibition of morphine tolerance and physical dependence by TRH may involve mechanisms other than those associated with opioid receptors,

Melanotropin Release Inhibiting Factor (MIF) and Its Analogs. The hormones of the posterior pituitary, vasopressin, and oxytocin and their analogs elicit a variety of actions on the CNS. Some of these actions include facilitation of conditioned avoidance behaviors in intact and hypophysectomized rats and have been reviewed (Bhargava 1987). Several of the analogs of vasopressin and oxytocin (50 μg/mouse) were shown to facilitate the development of tolerance and physical dependence on morphine in mice (Krivoy et al. 1974) and rats (1 μg/rat) (van Ree and de Wied 1976). Morphine was given by multiple injections, and analgesia was measured by the hot plate test. Some of this earlier work and inconsistencies have been summarized (Bhargava 1987). Studies have shown that the C-terminal peptides of oxytocin, Pro-Leu-Gly-NH_2 (MIF) and its synthetic analogs, cyclo (Leu-Gly), and other analogs were effective in inhibiting tolerance to morphine in mice and rats.

The majority of studies have been done with MIP and cyclo (Leu-Gly). Studies have been unable to show the facilitation of morphine tolerance with MIF. On the other hand, these peptides were demonstrated to block the tolerance to analgesic, cataleptic, hypothermic, and locomotor-depressant effects of morphine ß-endorphin and buprenorphine, as well as physical dependence on morphine. Further studies revealed that intragastric administration of MIF and cyclo (Leu-Gly) can block tolerance to the analgesic effect of morphine in the rat (Bhargava 1988; Bhargava and Ramarao 1989). Studies with Z-Pro-D-Leu on morphine dependence and with cyclo (Leu-Gly) on morphine tolerance have been confirmed by several investigators (Burton et al. 1991; Kovacs et al. 1981). In fact, Burton and colleagues (1991) have shown that cyclo (Leu-Gly) can also inhibit the tolerance to the respiratory-depressant action of morphine in the rat.

Although the mechanism by which MIF and its analogs produce their inhibiting action on tolerance to opioids is not evident, it is clear that they: (1) enhance the binding of dopamine agonists like ^{3}H-apomorphine in the brain, (2) do not affect the binding of μ,δ, or κ ligands to brain membranes, (3) inhibit the supersensitivity of dopamine receptors induced by morphine or ß-endorphin, (4) produce their effect through the formation of active metabolites, (5) antagonize the actions of κ opioid agonists, and (6) inhibit or reverse the changes in cyclic guanosine monophosphate (cGMP)-phosphodiesterase activity induced by morphine in the brain. Thus, even though the mechanisms in the opioid-induced tolerance/dependence process and in the action of peptides inhibiting this process are not understood, further studies are required to determine the actions of orally effective peptides.

Cholecystokinin (CCK) and Its Analogs. Considerable evidence suggests that CCK interacts with opioids in pain mechanisms. Although large doses (50 μg/kg and higher) of CCK induce naloxone-reversible analgesia (Jurna and Zetler 1981), small doses (3-16 μg/kg) of the drug inhibit the analgesic action of opioids (Faris et al. 1983) in rodents. The weak nonselective CCK antagonist proglumide and the potent CCK antagonist L-364,718 [1-methyl-3-(2-indoloyl) amino-5-phenyl-3H-1,4-benzodiazepine-2-one] potentiate morphine-induced analgesia and prevent the development of tolerance to morphine in the rat (O'Neill et al. 1989; Watkins et al. 1984). Similar enhancement of morphine analgesia and prevention of tolerance to systemically administered morphine has been demonstrated in the rat by CCK, receptor antagonist L-365,260 (Dourish et al. 1990).

Several lines of evidence suggest that CCK is released in response to activation of opioid receptors by morphine in the spinal cord. I.t. morphine causes the release of CCK from the spinal cord (Tang et al. 1984). The release of CCK is controlled selectively by δ opioid receptors (Ruiz-Gayo et al. 1992). Since the management of clinical pain by administering opioids by i.t. or epidural route has become important and tolerance develops to the analgesic effect of spinally administered opioid, the effect of i.t. administration of the CCK antagonist proglumide on the tolerance to morphine induced by i.t. injection in the rat has been studied (Kellstein and Mayer 1991). The spinal co-administration of lorglumide or proglumide for 6 days prevented the tolerance to i.t.-administered morphine in the rat. The tolerance induced by a higher dose of morphine (3 μg) required a higher dose of the CCK antagonists, whereas a lower dose of the antagonist was required when tolerance was induced by a 1-μg dose of the drug. Thus, CCK antagonists may prove to be useful adjuncts to opioids in the management of chronic pain.

Neurotransmitter Receptor System Modifiers

The role of several neurotransmitter receptor systems in morphine tolerance and abstinence has been reviewed (Bhargava, in press). They include serotonin, dopamine, opioid, and gamma amino butyric acid (GABA) receptors. However, the role they play is not entirely clear. Drugs modifying these receptor systems appear to modify analgesia, tolerance, and physical dependence on morphine. Thus, GABA antagonizes morphine analgesia and enhances morphine tolerance and physical dependence development. Bromocriptine, a specific dopamine D_2 agonist, potentiated morphine analgesia, suppressed the development of tolerance to the analgesic effect, and exacerbated withdrawal in morphine-dependent mice.

Adenosine receptors have been classified as A, or A, depending on whether they inhibit (A_1) or stimulate (A_2) the accumulation of cyclic AMP (cAMP) (van Calker et al. 1979). (-)-N^6-(R-phenylisopropyl)-adenosine (PIA), an adenosine A, agonist, produced analgesia in both the tail flick and the acetic acid writhing assays. The analgesic action of PIA was antagonized by caffeine, an antagonist in a noncompetitive manner. PIA potentiated morphine-induced analgesia, tolerance, and dependence. The effects of PIA were antagonized by caffeine. Thus, adenosine agonists facilitate the actions of morphine, whereas adenosine antagonists can antagonize them (Ahlijanian and Takemori 1985).

N-Methyl-D-Aspartate (NMDA) Receptor Antagonists

Recent evidence suggests that the excitatory amino acid (EAA) receptor systems (namely, NMDA, quisqualate, and kainate) may be involved in pain and opioid tolerance and dependence (OTD) mechanisms. These EAAs produce analgesia when microinjected into the periaqueductal gray matter (Jacquet 1988). The NMDA receptor antagonist MK-801 has been shown to produce no effect (Bhargava and Matwyshyn 1993; Marek et al. 1991) on morphine analgesia. However, it antagonizes κ opioid induced analgesia (Kest et al. 1992). Both kynurenic acid, a wide-spectrum EAA antagonist, and a noncompetitive NMDA receptor antagonist, MK-801, were shown to inhibit the development of tolerance to the analgesic effect of morphine in the rat (Marek et al. 1991). Both tolerance and physical dependence were attenuated by MK-801; however, high doses produced severe toxicity and death (Trujillo and Akil 1991). When injected once a day or twice a day, MK-801 (0.03 to 0.3 mg/kg, i.p.) inhibited the tolerance to the analgesic but not the hyperthermic effects of morphine. In once-a-day MK-801 treatment, there was no dose-dependent effect but, in twice-a-day treatment, it was dose dependent. However, in both cases increased mortality was observed with increasing doses of MK-801 administered in morphine-pelleted animals (Bhargava and Matwyshyn 1993).

Studies indicate that, in morphine-tolerant and abstinent rats, in the absence of glutamate and glycine, the binding of ^{3}H-MK-801 decreases modestly only in the cortex (Gudehithlu et al. 1994); however, the binding of ^{3}H-MK-801 in the presence of glutamate and glycine is decreased in some brain regions (Bhargava et al., in press). This suggests that, perhaps, the activity of endogenous glutamate and glycine is altered following the activation of the NMDA receptor.

Drugs Interfering With the Second Messenger Systems

Nitric Oxide Synthase (NOS) Inhibitors. It is clear from the previous section that NMDA receptor antagonist MK-801 attenuates tolerance to the analgesic action of morphine in the rat. Several of the NMDA effects are mediated via activation of NOS with subsequent release of nitric oxide (NO), which in turn increases the formation of cGMP (Bredt and Snyder 1992). NO has been recognized as a prominent neuronal second messenger, and its enzymatic formation from L-arginine has been demonstrated in cytosol obtained from rat forebrain synaptosomes (Knowles et al. 1989). Recently, N^{G}-nitro-L-arginine (NOArg), an

inhibitor of NOS, was shown to prevent the development of tolerance to morphine in mice (Kolesnikov et al. 1992). The same authors showed that NOArg also reduces morphine dependence but does not block tolerance to the κ opioid U-50,488H in mice (Kolesnikov et al. 1993). However, studies have clearly shown that N^G-monomethyl-L-arginine (NMMA) (2-8 mg/kg, i.p.) blocked the tolerance to the analgesic and hypothermic actions of U-50,488H in the mouse. The tolerance to U-50,488H was induced by injecting the drug (25 mg/kg, i.p.) twice a day for 4 days. By itself, NMMA did not alter the analgesic and hypothermic actions of U-50,488H in naive mice (Thorat et al. 1993).

Pertussis Toxin (PTX)-Role of GTP-Binding Proteins. The second messenger system most commonly associated with opioid receptors involves inhibition of adenylate cyclase (Sharma et al. 1975, 1977). Opioid receptor activation leads to opening of potassium channels in neurons of the CNS and inhibition of voltage-dependent calcium channels in primary cultures of dorsal root ganglion (North 1986). The control of ion channels and inhibition of adenylate cyclase are regulated by the G proteins (Dolphin 1987). PTX, which interferes with the G protein-dependent mechanism, has been shown to inhibit morphine analgesia when injected in the rat brain (Parolaro et al. 1990). Six days after the i.c.v. injection, PTX (0.5 μg/rat) decreased the analgesic response to morphine when given i.t., i.p., or in the periaqueductal gray matter. PTX also inhibited the development of physical dependence as evidenced by inhibition of naloxone-precipitated teeth chattering, rearing, and grooming in the rat (Parolaro et al. 1990). In dependence studies, two morphine pellets (75 mg) were implanted 3 days after i.c.v. pretreatment with PTX (1 μg/rat). The withdrawal was precipitated with naloxone (2 mg/kg, i.p.) 72 hours after the pellet implantation. These and earlier studies suggest that PTX-sensitive G proteins are necessary for signal transduction in a series of events leading to the production of morphine-induced analgesia or dependence.

DRUGS THAT MODIFY THE SYMPTOMS OF MORPHINE OR HEROIN ABSTINENCE SYNDROME

The desirable qualities of an opioid pharmacotherapeutic agent are that it should (1) be orally effective with high bioavailability, (2) have a long biological half-life generally greater than 24 hours, (3) have minimum side effects during chronic administration, and (4) have a high therapeutic index. In the following sections, the effects of some opioids and

nonopioid drugs on morphine abstinence syndrome in animals and humans will be described.

Drugs Related to Opioids

The use of clinical observation along with existing preclinical knowledge led to the development of a chronic pharmacotherapy for the treatment of opioid (heroin) dependence using the long-acting opioid methadone (Dole et al. 1966). After chronic administration of morphine or heroin, tolerance to euphoric and analgesic actions develops. Similarly, physical dependence also develops. Although the signs and symptoms of opioid abstinence syndrome are seen most prominently during the first 2 to 4 days after withdrawal (O'Brien et al. 1988), subtle signs and symptoms may be observed for 6 months or longer that may include depression and other abnormalities (Martin and Jasinsky 1969). The heroin addict typically self-administers the drug three to six times a day because it has a relatively short duration of action to achieve a euphoric feeling and to prevent narcotic withdrawal symptoms (Dole et al. 1966). Methadone was found to be orally active with a half-life in the range of 24-36 hours. It also reduces or eliminates drug craving and euphoria. Thus, it has been used as a once-a-day medication. A symptom that persisted in 50 percent of the subjects was increased sweating. A much longer acting (3-4 days) drug than methadone is L-cl-acetylmethadol (LAAM) which also is orally effective.

Endogenous Opioids and Their Analogs

Chronic administration of opioids, particularly of morphine or heroin, has been shown to be associated with alterations in the levels of endogenous opioid peptides in the brain regions, cerebrospinal fluid, plasma or serum, and other peripheral tissues of animals and humans. In general, there is a decrease in the tissue levels of Met-enkephalin, ß-endorphin, and dyn A(1-13) in morphine-tolerant and abstinent rats (Bhargava et al. 1989; Gudehithlu et al. 1991; Hollt et al. 1978; Przewloci et al. 1979; Rattan et al. 1992; Wesche et al. 1977); monkeys (Elsworth et al. 1986); and heroin-dependent human subjects (Clement-Jones et al. 1979; Ho et al. 1980; O'Brien et al. 1988; Volpe et al. 1986). Studies, therefore, have been undertaken to determine the effects of various opioid peptides and their analogs on the abstinence syndrome in morphine-dependent rodents, morphine-dependent monkeys, and heroin-dependent humans.

Natural and Synthetic Enkephalins. Studies have shown that Met- and Leu-enkephalin when injected i.c.v. inhibited the naloxone-precipitated withdrawal jumping response in morphine-dependent mice (Bhargava 1977, 1978*b*; Leybin et al. 1976). Further studies revealed that i.c.v. injections of two synthetic analogs of enkephalins, D-Ala^2, Met'-enkephalinamide and D-Met^2-Pro^5-enkephalinamide, and morphine inhibited naloxone-precipitated withdrawal jumping and hypothermia, as well as hypothermia seen during abrupt withdrawal in morphine-dependent mice. On a molar basis, D-Met^2-Pro^5-enkephalinamide and D-Ala^2-Met^5-enkephalinamide were 23 and 3 times more potent, repectively, than morphine in inhibiting morphine abstinence syndrome (Bhargava 1980). The effects of two enzyme-resistant analogs of enkephalin, namely FK-33824 and metkephamid, administered either i.c.v. or s.c. to morphine-dependent rhesus monkeys also have been determined. Rhesus monkeys were maintained on morphine (3 mg/kg, s.c.) every 6 hours for at least 90 days, and then the morphine was withdrawn for 12-16 hours. Both compounds suppressed the abstinence syndrome in a dose-related fashion. FK-33824 was 100 times more potent in suppressing abstinence syndrome when given by i.c.v. route than by s.c. route, whereas metkephamid was 2,000 times more potent when administered centrally in comparison to peripheral administration. Morphine was only 5 times more potent by i.c.v. route than S.C. injection. I.c.v.-administered morphine and FK-33824 suppressed withdrawal signs for 5 and 13 hours, respectively (Gmerek et al. 1983). I.v. administration of [D-Ala^2, D-Leu^5] enkephalin (DADLE) (60 µg/kg) has been shown to significantly inhibit withdrawal symptoms in heroin addicts for an hour; however, it also produced many side effects, which included weakness of limbs, tightness of chest, precordial pain, headache, and heat sensation (Wen et al. 1984).

Natural and Synthetic Dynorphins. Dyn A(1-13) injected i.v. or i.t. but not i.c.v. inhibited wet dog shakes, yawns, abdominal stretches, and ptosis observed during abrupt withdrawal in morphine-dependent rats (Green and Lee 1988). Similar effects of dyn A(1-13) have been observed in morphine-dependent rhesus monkeys (Aceto et al. 1982). Dyn A(1-13) injected i.v. (60 µg/kg) suppressed the withdrawal symptoms in heroin addicts, which were associated with side effects such as feeling of warmth, giddiness, dizziness, and precordial formication (Wen and Ho 1982; Wen et al. 1984). Although dyn A(1-10) amide has been claimed to be a potent and selective agonist of dyn A(1-13), in heroin addicts dyn A(1-13) was found to be more potent than dyn A(1-10) amide (Wen et al. 1984). In addition, i.t. administration

of dyn A(1-13) has been reported to induce paralysis in the rat (Herman and Goldstein 1985).

ß-Endorphin. ß-endorphin administered i.c.v. in doses of 0.09-0.17 µg/mouse suppressed the withdrawal jumping induced by abrupt withdrawal of morphine from morphine pellet-implanted mice (Tseng et al. 1976). In this design, morphine pellets were removed from the mice. Five to six hours later, the spontaneous jumpers and nonjumpers were given the test dose of ß-endorphin. No other withdrawal sign was monitored. Sixty µg/kg of ß-endorphin injected i.v. also attenuated the withdrawal symptoms in heroin addicts for 45 minutes. Such an inhibition was associated with euphoria in some patients (Wen et al. 1984).

Inhibitors of Peptidases and Enkephalinases

The endogenous and exogenous enkephalins are degraded by enzymes termed “enkephalinases A and B" and aminopeptidases that are zinc-containing metallozymes (Schwartz 1983). Inhibition of these enzymes presumably should increase the tissue levels of the enkephalins and other opioid peptides. The effects of several specific and nonspecific inhibitors of enkephalinases and peptidases on the opioid withdrawal syndrome has been studied. I.c.v. administration of bacitracin and aprotinin decreased the behavioral withdrawal score, as well as the epileptogenic expression in rats made dependent by increasing doses of morphine for 9 days and then precipitating the withdrawal by naloxone (Pinsky et al. 1982). Another enkephalinase inhibitor, phosphoramidon (50-200 µg, i.c.v.), suppressed naloxone-precipitated withdrawal jumping and wet dog shakes while forelimb shakes were potentiated in acute and chronic morphine-dependent mice (Dzoljic et al. 1986). Injection of another enkephalinase inhibitor, thiorphan (40 µg), i.c.v. or into the periaqueductal gray matter inhibited the withdrawal precipitated by naloxone (4 mg/kg, i.p.) in rats made dependent on morphine by implantation of three 75 mg morphine pellets for 3 days (Haffmans and Dzoljic 1987). Thiorphan was injected 10 minutes prior to the injection of naloxone. The symptoms that were suppressed included digging, head hiding, diarrhea, teeth chattering, wet dog shakes, grooming, rearing, and paw tremor. In general, thiorphan injected into the periaquecductal gray region was’better than i.c.v. in inhibiting the symptoms of the abstinence syndrome (Haffmans and Dzoljic 1987). Similar effects have been observed with other mixed enkephalinase inhibitors, kelatorphan [(R)-3-(N-hydroxy-carboxamide-2-benzyl propanoyl)-L-alanine] and RB 38A

[(R)-3-(N-hydroxycarboxamido-2-benzyl propanoyl)-L-phenylalanine] (i.e., inhibition of jumping, chewing, and teeth chattering in naloxone-precipitated withdrawal in morphine-dependent rats). The body weight loss was unaffected by enkephalinase inhibitors (Maldonado et al. 1989). Finally, the i.p. administration of orally effective enkephalinase inhibitors acetorphan (2.5-20 mg/kg) and SCH 34826 (15-120 mg/kg) decreased the severity of the naloxone-precipitated withdrawal syndrome in morphine-dependent mice and rats (Dzoljic et al. 1992).

Nonopioid Peptides

TRH and Its Analogs. TRH administered i.c.v. (1-50 µg per mouse) inhibited naloxone-precipitated withdrawal jumping in mice rendered dependent on morphine by pellet implantation. Similarly, hypothermic response observed during abrupt or naloxone-precipitated withdrawal was inhibited by i.c.v. administration of TRH (Bhargava et al. 1983). Such results are consistent with the observations of Morley and colleagues (1980), who demonstrated that during withdrawal the brain concentration of TRH decreases. Since TRH has a short half-life, the effects of its more stable analogs on morphine abstinence syndrome were determined. The naloxone-precipitated abstinence syndrome also was inhibited by cyclo (His-Pro), a metabolite of TRH in morphine-dependent mice (Bhargava et al. 1983). Effects similar to TRH were observed with two analogs, L-N-(2-oxo-piperidin-6-glycarbonyl)-L-histidyl-L-thiazolidine-4-carboxamide (MK-771) and γ-butyrolactone-4-carboxyl-histidyl-prolineamide (DN-1417) (Bhargava and Matwyshyn 1985).

Drugs Related to Neurotransmitter Systems and Second Messenger Systems

A number of neurotransmitter systems have been implicated in the expression of various symptoms of withdrawal from morphine. They include norepinephrine, dopamine, acetyl choline, adenosine, and NMDA receptors and some of their coupled second messenger systems. Involvement of each of these systems will be described briefly.

Noradrenergic System. Considerable evidence suggests that opioid withdrawal syndrome may involve changes in central noradrenergic activity. Hypersensitivity to noradrenergic neurons has been established in the cerebral cortex of morphine-dependent rats (Llorens et al. 1978), as evidenced by increased B_{max} of ^{3}H-dihydroalprenolol and increased accumulation of cAMP in response to norepinephrine and isoproterenol.

Similarly, increased activity of noradrenergic neurons has been seen in the locus coeruleus of morphine-dependent rats (Aghajanian 1978) and primates (Redmond and Huang 1982). Increased α_2-adrenergic receptors have been observed in chronically morphine-treated rats (Hamburg and Tallman 1981). Hyperadrenergic activity in methadone-dependent human subjects given naltrexone has been evidenced by the increased concentration of plasma MHPG, a metabolite of norepinephrine (Chamey et al. 1984). Thus, both clinical and preclinical studies have provided evidence for the hyperadrenergic activity, and drugs have been used that modify adrenergic activity to treat the withdrawal symptoms in opioid-dependent subjects. Clonidine, an α_2–receptor agonist, injected i.p. or i.c.v. inhibited naloxone-precipitated wet dog shakes and escape attempts in morphine-dependent rats (Tseng et al. 1975). Clonidine and guanfacine inhibit both behavioral and autonomic components of morphine withdrawal in the rat (Buccafusco et al. 1984). Clonidine also eliminated objective and subjective symptoms of opioid withdrawal in 11 addicts. The patients had been addicted to opioids for 6-10 years and to methadone for 6-60 months (Gold et al. 1979).

Dopaminergic Systems. Although five types of dopamine receptors have been identified and cloned (O'Dowd 1993), the majority of the studies on OTD or abstinence have involved D_1 and D_2 receptors. Dopamine D_1 receptors are linked to adenylate cyclase, whereas D_2 receptors are not. Although it has been known for several years that behavioral supersensitivity to dopamine agonists is observed in morphine- and ß-endorphin-treated rodents, it was not clear which specific dopamine receptors were involved. Recent studies have shown that MTD and abstinent rats show differential changes in the central dopamine D_1 and D_2 receptors. In nonabstinent MTD rats, both D_1 and D_2 receptors are unaltered in brain regions and spinal cord when labeled with ^{3}H-SCH 23390 and ^{3}H-spiroperidol, respectively. In the abstinent rats, the binding (B_{max} value) of ^{3}H-SCH 23390 is increased in hypothalamus, corpus striatum, and spinal cord and decreased in amygdala, whereas the binding of ^{3}H-spiroperidol is unaffected in brain and spinal cord. However, the behavioral response to both D_1 and D_2 agonists, SKF 38393 and bromocryptine, respectively, are enhanced in morphine-abstinent rats (Bhargava, in press). In general, dopamine agonists like apomorphine enhance, whereas dopamine blockers like haloperidol and butaclamol inhibit wet dog shakes and aggressive behavior observed during withdrawal in morphine-dependent rats. Haloperidol also inhibits withdrawal symptoms in human heroin addicts (Karkalas and Lal 1973).

Cholinergic Systems. Cholinergic drugs that are both direct acting as well as indirect acting such as physostigmine, an acetylcholinesterase inhibitor, suppress the naloxone-induced withdrawal jumping response in morphine-dependent mice (Bhargava and Way 1972). Similarly, pilocarpine reduced wet dog shakes and aggression but enhanced diarrhea and weight loss in rats made dependent by multiple injections of morphine. Pretreatment with atropine and methyl-scopolamine blocked only the diarrhea induced by pilocarpine. In addition, the brain concentration of acetylcholine is decreased during abstinence from morphine (Bhargava and Way 1975).

Benzodiazepine Receptor Agonists and Antagonists. The discovery of the high-affinity stereospecific benzodiazepine receptors in the CNS has led to the clinical use of benzodiazepine receptor agonists and antagonists in many CNS-related disorders. Even before this discovery, benzodiazepines, which are anxiolytic agents, were used in the treatment of opioid abstinence syndrome. Thus, prazepam and chlordiazepoxide have been used to suppress symptoms of the opioid withdrawal syndrome in adult narcotic addicts (Drummond et al. 1986, 1989; Sugerman et al. 1971), whereas diazepam has been used in neonates undergoing withdrawal (Nathenson et al. 1971). Administration of flunitrazepam, a benzodiazepine agonist, decreases jumping and wet dog shakes in morphine-dependent mice (Gibert-Rahola et al. 1988; Valverde et al. 1992) and rats (Baldino et al. 1979; Maldonado et al. 1991). Naloxone-precipitated withdrawal jumping and wet dog shakes in morphine-dependent mice was increased by R015-4513, a partial inverse benzodiazepine agonist, and flumazenil, a benzodiazepine antagonist (Valverde et al. 1992).

Adenosine Receptor Agonists. The effects of the adenosine receptor A_1 agonist PIA, the mixed adenosine A_1 and A_2 agonist adenosine-5′-ethylcarboxamide (NECA), and the adenosine A_2 selective agonist 2-(phenylamino) adenosine (CV-1808) on naloxone-precipitated withdrawal has been studied in morphine-dependent rats. PIA and NECA produced dose-related decrease in the total amount of fecal matter, and CV-1808 had a similar effect, but the effect was not dose dependent. Behaviors like paw shakes, body shakes, teeth chattering, and jumping were inhibited by PIA and NECA. The latter was more potent than PIA. CV-1808 caused a reduction only in the incidence of teeth chattering. Locomotor activity was reduced by all adrenosinergic agonists (Dionyssopoulos et al. 1992). Chronic treatment of rats with morphine

by using multiple injections has been shown to down-regulate adenosine A_1 receptors in the spinal cord but not in cortex (Tao and Liu 1992).

Calcium Channel Blockers. Considerable evidence suggests that morphine exhibits its analgesic activity, at least in part, through the inhibition of calcium influx, thereby reducing the release of transmitters. Using the acetic acid writhing test, Ohnishi and colleagues (1988) demonstrated that the analgesic effect of nifedipine, a calcium channel blocker, was decreased in morphine-tolerant mice. Thus, calcium influx may be one of the mechanisms of the analgesic action of morphine, and chronic administration of morphine produces an increase of calcium entry. Increased density of ^{3}H-nitrendipine binding sites has been observed in the mouse and rat brain (Ramkumar and El-Fakahany 1984, 1988) when the animals were treated chronically with morphine, suggesting an increase in the number of calcium channels. In morphine-dependent rats, i.p. administration of verapamil and flunarizine prevented diarrhea and weight loss but not jumping observed during withdrawal. Verapamil reduced the incidence of ptosis. I.c.v. administration of verapamil (160 μg/kg) reduced the body weight loss and jumping response without modifying diarrhea or ptosis. Thus, both central and peripheral mechanisms are important in the inhibition of morphine abstinence syndrome by calcium channel blockers (Baeyens et al. 1987).

Similar effects have been demonstrated with the isomers of diltiazem, d-cis isomer being more potent that I-cis isomer in inhibiting naloxone-precipitated withdrawal in morphine-dependent mice (Caro et al. 1988). Verapamil, diltiazem, and nicardipine also decreased forepaw tremor, body weight loss, and jumping in mice acutely dependent on morphine. On the other hand, calcium agonist Bay K 8644 increased forepaw tremor and body weight loss (Barrios and Baeyens 1991). In another study, verapamil and nimodipine were reported to inhibit naloxone-precipitated withdrawal symptoms in morphine-dependent rats. These effects were produced by an action independent of opioid receptors since both agents did not displace ^{3}H-naloxone from its binding sites. Nimodipine pretreatment also antagonized the decreases in norepinephrine contents and increases in MHPG levels in the cortex, brain stem, and hippocampus induced by the abstinence syndrome (Bongianni et al. 1986).

NMDA Receptor Antagonists. In mice rendered dependent on morphine by pellet implantation, MK-801 (0.1 mg/kg, i.p.) did not alter the jumping response, but 1.0 mg/kg dose of MK-801 significantly reduced the jumping response precipitated with 0.05 mg/kg of naloxone.

In mice made dependent by multiple injections of morphine for 9 days, MK-801 (0.17 mg/kg, i.p.) abolished jumping behavior induced by naloxone (1 mg/kg, i.p.). On the other hand, 0.054 mg/kg of MK-801 increased jumping behavior (Marquis et al. 1991). Because of these complex interactions, studies were undertaken to determine the effect of MK-801 on naloxone-precipitated withdrawal in mice made dependent by morphine (75 mg) pellet implantation (Thorat et al. 1994). Injection of MK-801 (0.03, 0.1, and 0.3 mg/kg, i.p.) given 30 minutes before naloxone (50 µg/kg, s.c.) did not modify stereotypical jumping behavior, body weight loss, body temperature, or the formation of fecal boli (Thorat et al. 1994).

The effects of MK-801, a noncompetitive antagonist that acts by blocking the ion channel, and LY 274614, a competitive NMDA receptor antagonist that acts through an action at the glutamate recognition site, on morphine abstinence syndrome have been determined in the rat (Rasmussen et al. 1991). MK-801 (0.1 and 1.0 mg/kg) pretreatment significantly suppressed teeth chattering, erections, ptosis, chewing action, wet dog shakes, weight loss, lacrimation, and diarrhea induced by naltrexone in morphine-dependent rats. The symptoms that were unaffected included writhes, jumping, and salivation. In fact, MK-801 at 0.1 mg/kg increased the number of wet dog shakes. Similarly, LY 274614 suppressed the same behaviors as did MK-801 but did not affect symptoms like writhing, jumping, wet dog shakes, salivation, and lacrimation. MK-801 but not LY 274614 pretreatment produced phencyclidine-like behaviors, including head weaving, locomotion, and falling. Thus, studies in both mice and rats indicate that NMDA receptor antagonists inhibit some symptoms of the antagonist-induced abstinence syndrome but do not affect stereotypical jumping responses.

Nitric Oxide Synthase (NOS) Inhibitors. Recent studies indicate that a NOS inhibitor like NMMA inhibits the stereotypical jumping response but does not affect other withdrawal signs in morphine-dependent mice (Thorat et al. 1994). Similar effects have been observed with NOArg, another inhibitor of NOS on morphine abstinence syndrome in mice (Kolesnikov et al. 1993).

Natural Products

Marijuana Constituents. The chemical constituents of marijuana (Cannabis sativa), known as cannabinoids, have been shown to modify the symptoms of antagonist-induced withdrawal in morphine-dependent

rodents. Several studies have shown that morphine and 1-trans-Δ^9-tetrahydrocannabinol (Δ^9-THC) share many pharmacological properties, including analgesia, hypothermia, respiratory depression, locomotor depression, and tolerance development, even though the mechanisms of action of these two compounds are quite different. More recently, a bidirectional cross-tolerance between morphine and Δ^9-THC has been demonstrated in mice (Thorat and Bhargava, in press). The withdrawal induced wet dog shakes and defecation responses were inhibited by Δ^9-THC in morphine-dependent rats. In morphine-dependent mice, administration of Δ^9-THC (2.5 to 10 mg/kg, i.p.) inhibited the stereotypical jumping response, defecation, and rearing behavior. Δ^8-tetrahydrocannabinol (Δ^8-THC), 11 -hydroxy-Δ^8-THC, cannabidiol, and cannabinol also inhibited the responses. Δ^9-THC was the most potent agent, whereas cannabinol was the least potent. Further studies on the time course of the effects of cannabinoids revealed that Δ^9-THC at 10 mg/kg was effective for 24 hours in inhibiting the jumping response, whereas Δ^8-THC and 11-hydroxy-Δ^8-THC were effective for only 2 hours. This inhibition in naloxone-precipitated jumping response by cannabinoids appeared to be specific since the vertical jumping syndrome induced by co-administration of amphetamine and l-dopa was not affected by cannabinoids (Bhargava 1978*c*).

Immunomodulators

Interferon. Substantial evidence exists to suggest that the CNS can influence the immune function and vice versa. It also is known that chronic morphine treatment not only influences the CNS but also the immune function (Bhargava 1991*b*; Bhargava et al. 1994*b*). Morphine has been shown to decrease the circulating levels of interferon, a naturally occurring protein that stimulates natural killer cell activity, decreases the number of circulating B lymphocytes, and inhibits T cell proliferative responses (Hung et al. 1973). Injection of recombinant leukocyte A interferon (150 units/g, i.p.) 1 hour prior to naloxone reduced or eliminated wet dog shakes, teeth chattering, fecal boli formation, hyperactivity, exploring behavior, and diarrhea in morphine-dependent rats (Dafny 1983). Although the mechanism by which interferon inhibits morphine abstinence syndrome is not known, it has structural similarity to adrenocorticotropic hormone$_{1\text{-}13}$ ($ACTH_{1\text{-}13}$) and ß-endorphin (Blalock and Smith 1980). Finally, human leukocyte interferon has been shown to bind to opioid receptors in vitro (Blalock and Smith 1981).

Cyclosporine. Effects similar to interferon have been observed with cyclosporine (15 mg/kg, i.p.) injected 2 hours prior to naloxone (1 mg/kg, i.p.) in morphine-dependent rats (Dafny et al. 1985).

SUMMARY AND CONCLUSION

In summary, the effects of drugs affecting the MTD and abstinence syndrome in mice, rats, monkeys, and humans have been described. Although μ opioid antagonists block tolerance and dependence on morphine, they also antagonize the analgesic response to morphine and, therefore, may not be useful clinically in inhibiting the MTD process. On the other hand, Q-selective opioid antagonists do not modify morphine analgesia but block the MTD process and, therefore, may have therapeutic potential. Neuropeptides like TRH, MIF, and cyclo (Leu-Gly) appear to produce similar results although their mechanism of action may be quite different. Cyclo (Leu-Gly) produces its action on oral administration and, therefore, further studies are warranted with this peptide. The CCK_B receptor antagonist enhances morphine analgesia and blocks tolerance to morphine but has no effect on the development of dependence on morphine. EAA antagonists like MK-801 block tolerance to the analgesic effect of morphine selectively but are quite toxic. Therefore, newer, less toxic agents like LY 274,614 need to be developed.

Further studies are necessary with NOS inhibitors that inhibit both tolerance to and physical dependence on morphine. Exploration of agents from natural products like ginseng constituents also may prove to be fruitful. The opioid abstinence syndrome is modified by a variety of agents. Substitution therapy with orally acting agents with long duration of action like methadone and LAAM is favored, with methadone and LAAM being the drugs of choice. Since, in general, in morphine- or heroin-addicted subjects, the levels of endogenous opioids in the CNS decrease, one can see the appearance of symptoms of the withdrawal syndrome, replenishing the stores of endogenous opioids to suppress the symptoms.

In this regard, preclinical and clinical studies suggest that dyn A(1-13) and related compounds may be useful. However, they will not be effective orally. Additionally, the toxicity of dyn A(1-13) must be evaluated carefully. On the other hand, orally effective enkephalinase inhibitors, which apparently increase the levels of endogenous opioids

may prove to be good therapeutic agents. Drugs that inhibit noradrenergic and dopaminergic activity (i.e., clonidine and antipsychotic agents), increase central cholinergic activity, or both, may be useful for management of opioid withdrawal syndrome. Similarly, NMDA antagonists, NOS inhibitors and benzodiazepine anxiolytic also may be useful adjuncts. Finally, the use of immunomodulators and calcium channel blockers in the management of opioid abstinence syndrome still need to be explored in further detail.

REFERENCES

Abdelhamid, E.E.; Sultana, M.; Portoghese, P.S.; and Takemori, A.E. Selective blockage of δ opioid receptors prevents the development of morphine tolerance and dependence in mice. *J Pharmacol Exp Ther* 258:299-303, 1991.

Aceto, M.D.; Dewey, W.L.; Chang, J.K.; and Lee, N.M. Dynorphin (1-13): Effects in nontolerant and morphine-dependent rhesus monkeys. *Eur J Pharmacol* 83: 139-142, 1982.

Aceto, M.D.; Dewey, W.L.; Portoghese, P.S.; and Takemori, A.E. Effect of ß-funaltrexamine (P-FNA) on morphine dependence in rats and monkeys. *Eur J Pharmacol* 123:387-393,1986.

Aghajanian, G.K. Tolerance locus coetuleus neurons to morphine and suppression of withdrawal responses by clonidine. *Nature* 276:186-188, 1978.

Ahlijanian, M.K., and Takemori, A.E. Effects of (-)-N^6-(R-phenylisopropyl) adenosine (PIA) and caffeine on nociception and morphine-induced analgesia, tolerance and dependence in mice. *Eur J Pharmacol* 112:171-179, 1985.

Baeyens, J.M.; Esposito, E.; Ossowska, G.; and Samanin, R. Effects of peripheral and central administration of calcium channel blockers on the naloxone-precipitated abstinence syndrome in morphine-dependent rats. *Eur J Pharmacol* 137:9-13, 1987.

Baldino, F.; Cowan, A.; Geller, E.B.; and Adler, M.W. Effects of antipsychotic and antianxiety drugs on morphine abstinence syndrome in rats. *J Pharmacol Exp Ther* 208:63-66, 1979.

Barrios, M., and Baeyens, J.M. Differential effects of L-type calcium channel blockers and stimulants on naloxone-precipitated withdrawal in mice acutely dependent on morphine. *Psychopharmacology* 104:397-403, 1991.

Bhargava, H.N. Opioid-like action of methionine-enkephalin in inhibiting morphine abstinence syndrome. *Eur J Pharmacol* 41:81-84, 1977.

Bhargava, H.N. The effects of naltrexone on the development of physical dependence on morphine. *Eur J Pharmacol* 50:193-202, 1978*a* .

Bhargava, H.N. New in vivo evidence for narcotic agonistic property of leucine-enkephalin. *J Pharm Sci* 67:136-137, 1978*b*.

Bhargava, H.N. Potential therapeutic application of natural and synthetic cannabinoids. *Gen Pharmacol* 9:1195-213, 1978*c*.

Bhargava, H.N. Comparative effects of synthetic enkephalinamides and morphine on abstinence responses in morphine-dependent mice. *Pharmacol Biochem Behav* 12:645-649, 1980.

Bhargava, H.N. Peptides as drugs in the treatment of opioid addiction. In: Rapaka, R.S., and Hawks, R.L., eds. *Opioid Peptides Molecular Pharmacology Biosynthesis and Analysis.* National Institute on Drug Abuse Research Monograph 70. DHHS Pub. No. (ADM)87-1455. Washington, DC: Supt. of Docs., U.S. Govt. Print. Off., 1987. pp. 337-365.

Bhargava, H.N. Intragastric administration of cyclo (Leu-Gly) inhibits the development of tolerance to the analgesic effect of morphine in the rat. *Life Sci* 43:187-192, 1988.

Bhargava, H.N. Multiple opiate receptors of brain and spinal cord in opiate addiction. *Gen Pharmacol* 22:767-772, 1991*a*.

Bhargava, H.N. Opioid systems and the immune function. In: Plotnikoff, N.P.; Murgo, A.; Faith, R.E.; and Wybran, J., eds. *Stress and Immunity*. Boca Raton, FL: CRC Press Inc., 1991*b* pp. 329-342.

Bhargava, H.N. Diversity of agents which modify opioid tolerance, physical dependence, abstinence syndrome and self-administrative behavior. *Pharmacol Rev,* in press.

Bhargava, H.N., and Matwyshyn, G.A. Comparative effects of thyrotropin releasing hormone, MK-771 and DN-1417 on morphine abstinence syndrome. *Psychophamacology* 87:141-146, 1985.

Bhargava, H.N., and Matwyshyn, G.A. Dizocilpine (MK-801) blocks the tolerance to the analgesic but not to the hyperthermic effect of morphine in the rat. *Pharmacology* 147:344-350, 1993.

Bhargava, H.N.; Matwyshyn, G.A.; and Ramarao, P. The effect of U-50,488H, aκ-opioid receptor agonist on tolerance to the analgesic and hyperthermic effects of morphine in the rat. *Gen Pharmacol* 22:429-434, 1991.

Bhargava, H.N.; Matwyshyn, G.A.; Gerk, P.M.; Bozek, P.S.; Bailey, M.D.; Ko, K.H.; Sinko, R.J.; and Thorat, S.N. Effect of naltrexone pellet implantation on morphine tolerance and physical dependence in the rat. *Gen Pharmacol* 25(1):149-155, 1994*a*.

Bhargava, H.N., and Ramarao, P. Comparative effects of Pro-Leu-Gly-NH, and cyclo (Leu-Gly) administered orally on the development of tolerance to the analgesic effect of morphine in the rat. *Peptides* 10:767-771, 1989.

Bhargava, H.N.; Ramarao, P.; Gulati, A.; Gudehithlu, K.P.; and Tejwani, G.A. Methionine-enkephalin and ß-endorphin levels in spleen and thymus gland of morphine tolerant-dependent and abstinent rats. *Life Sci* 45:2529-2537, 1989.

Bhargava, H.N.; Reddy, P.L.; and Gudehithlu, K.P. Down-regulation of N-methyl-D-aspartate (NMDA) receptors of brain regions and spinal cord of rats treated chronically with morphine. *Gen Pharmacol,* in press.

Bhargava, H.N.; Thomas, P.T.; Thorat, S.N.; and House, R.V. Effect of morphine tolerance and abstinence on the cellular immune function. *Brain Res* 642:1-10, 19943.

Bhargava, H.N.; Yousif, D.J.; and Matwyshyn, G.A. Interactions of thyrotropin releasing hormone, its metabolites and analogues with endogenous and exogenous opioids. *Gen Pharmacol* 14:565-570, 1983.

Bhargava, H.N., and Way, E.L. Acetylcholinesterase inhibition and morphine effects in morphine tolerance and dependent mice. *J Pharmacol Exp Ther* 183:3 1-40, 1972.

Bhargava, H.N., and Way, E.L. Brain acetylcholine and choline changes following acute and chronic morphinization and during withdrawal. *J Pharmacol Exp Ther* 194:65-73, 1975.

Blalock, J.E., and Smith, E.M. Human leukocytic interferon: Structural and biological relatedness to adrenocorticotropic hormone and endorphins. *Proc Natl Acad Sci* U *S A* 77:5972-5974, 1980.

Blalock, J.E., and Smith, E.M. Human leukocytic interferon (HulFN-α): Potent endorphin like opioid activity. *Biochem Biophys Res Commun* 101:472-478, 1981.

Bongianni, F.; Carla, V.; Moroni, F.; and Pellegrini-Giampietro, D.E. Calcium channel inhibitors suppress the morphine withdrawal syndrome in rats. *Br J Pharmacol* 88:561-567, 1986.

Bredt, D.S., and Snyder, S.H. Nitric oxide, a novel neuronal messenger. *Neuron* 8:3-11, 1992.

Buccafusco, J.J.; Marshall, D.C.; and Tarner, R.M. A comparison of the inhibitory effects of clonidine and guanfacine on the behavioral and autonomic components of morphine withdrawal in rats. *Life Sci* 35:1401-1408, 1984.

Burton, C.K.; Ho, I.K.; and Hoskins, B. Effect of cyclo (Leu-Gly) on cyclic GMP-phosphodiesterase activity changes associated with development of tolerance to morphine induced antinociception, catalepsy, respiratory depression and mydriasis. *J Pharmacol Exp Ther* 258:871-876, 1991.

Buscher, H.H.; Hill, R.C.; Romer, D.; Cardinaux, F.; Closse, A.; Hauser, D.; and Pless, J. Evidence for analgesic activity of enkephalin in the rat. *Nature* 261:423-425, 1976.

Caro, G.; Barrios, M.; and Baeyens, J.M. Dose-dependent and stereoselective antagonism by diltiazem of naloxone-precipitated morphine abstinence after acute morphine-dependence in vivo and in vitro. *Life Sci* 43:1523-1527, 1988.

Chapman, D.B.; Hu, J.; and Way, E.L. Methionine-enkephalin antagonism and endorphin potentiation of narcotic induced analgesia. *Eur J Pharmacol* 65:369-371, 1980.

Charney, D.S.; Redmond, D.E.; Galloway, M.P.; Kleber, H.D.; Heninger, G.R.; Murberg, M.; and Roth, R.H. Naltrexone precipitated opioids withdrawal in methadone addicted human subjects: Evidence for adrenergic hyperactivity. *Life Sci* 35:1263-1272, 1984.

Clement-Jones, V.; McLaughlin, L.; Lowry, P.J.; Besser, G.M.; Rees, L.H.; and Wen, H.L. Acupuncture in heroin addicts: Changes in met-enkephalin and ::-endotphin in blood and cerebrospinal fluid. *Lancet* 2:380-382, 1979.

Dafny, N. Modification of morphine withdrawal by interferon. *Life Sci* 32:303-305, 1983.

Dafny, N.; Wagle, V.G.; and Drath, D.B. Cyclosporine alters opiate withdrawal in rodents. *Life Sci* 36:1721-1726, 1985.

Dionyssopoulos, T.; Hope, W.; and Coupar, I.M. Effect of adenosine analogues on the expression of opioid withdrawal in rats. *Pharmacol Biochem Behav* 42:201-206, 1992.

Dole, V.P.; Nyswander, M.E.; and Kreek, M.J. Narcotic blockade. *Arch Intern Med* 118:304-309, 1966.

Dolphin, A.C. Nucleotide binding protein in signal transduction and disease. *Trends Neurosci* 10:53-57, 1987.

Dourish, C.T.; O'Neill, M.F.; Coughlan, J.; Kitchener, S.J.; Hawley, D.; and Iversnen, S.D. The selective CCK-B receptor antagonist L-365,260 enhances morphine analgesia and prevents morphine tolerance in the rat. *Eur J Pharmacol* 176:35-44, 1990.

Drummond, D.C.; Taylor, J.A.; and Mullin, P.I. Replacement of a prescribing service by an opioid free day programme in a Glasgow drug clinic. *Br J Addict* 81:559-565, 1986.

Drummond, D.C.; Turkington, D.; Rahman, M.Z.; Mullin, J.; and Jackson, P. Chlordiazepoxide vs. methadone in opioid withdrawal: A preliminary double blind trial. *Drug Alcohol Depend* 23:63-72, 1989.

Dzoljic, M.R.; Boksanska, A.; Korenhof, A.M.; Kaplan, C.D.; Dzoljic, M.; Rupresht, J.; Zijlstra, F.N.; Brinkman, E.C.A.; and Cappendijk, S.L.T. The effects of orally active enkephalinase inhibitors on morphine withdrawal syndrome. *Neuroreport* 3:637-640, 1992.

Dzoljic, M.R.; Rademaker, B.; Poel-Heisterkamp, A.L.; Ukponmwan, O.E.; and Haffmans, J. Enkephalinase inhibition suppresses naloxone-induced jumping in morphine-dependent mice. *Arch Int Pharmacodyn Ther* 283:222-228, 1986.

Elsworth, J.D.; Redmond, D.E., Jr.; and Roth, R.H. Effect of morphine treatment and withdrawal on endogenous methionine- and leucine-enkephalin levels in primate brain. *Biochem Pharmacol* 35:3415-3417, 1986.

Fat-is, P.L.; Kamisaruk, B.R.; Watkins, L.R.; and Mayer, D.J. Evidence for the neuropeptide cholecystokinin as an antagonist of opioid analgesia. *Science* 219:310-312, 1983.

Fukagawa, Y.; Katz, J.L.; and Suzuki, T. Effects of a selective κ-opioid agonist, U-50,488H on morphine dependence in rats. *Eur J Pharmacol* 170:47-51, 1989.

Gibert-Rahola, J.; Maldonado, R.; Mica, J.A.; Leonsequi, I.; and Saavedra, M.C. Comparative studies in mice of flunitrazepam vs. diazepam on morphine withdrawal syndrome. *Prog Neuropsychopharmacol Biol Psychiatry* 12:927-933, 1988.

Gmerek, D.E.; Katz, J.L.; France, C.P.; and Woods, J.H. Systemic and intracerebroventricular effects of opioid peptides in withdrawn morphine-dependent rhesus monkeys. *Life Sci* 33:361-364, 1983.

Gold, M.S.; Redmond, D.E., Jr.; and Kleber, H.D. Noradrenergic hyperactivity in opioid withdrawal. *Am J Psychiatry* 136:100-102, 1979.

Green, P.G., and Lee, N.M. Dynorphin A-(1-13) attenuates withdrawal in morphine-dependent rats: Effect of route of administration. *Eur J Pharmacol* 145:267-272, 1988.

Gudehithlu, K.P.; Reddy, P.L.; and Bhargava, H.N. Effect of morphine tolerance and abstinence on the binding of ^{3}H-MK-801 to rat brain regions and spinal cord of the rat. *Brain Res* 639:269-274, 1994.

Gudehithlu, K.P.; Tejwani, G.A.; and Bhargava, H.N. ß-Endorphin and methionine-enkephalin levels in discrete brain regions, spinal cord, pituitary gland and serum of morphine tolerant-dependent and abstinent rats. *Bruin Res* 553:284-290, 1991.

Haffmans, J., and Dzoljic, M.R. Inhibition of enkephalinase activity attenuates naloxone-precipitated withdrawal symptoms. Gen *Pharmacol* 18:103-105, 1987.

Hamburg, M., and Tallman, J.F. Chronic morphine administration increases the apparent number of α_2-adrenergic receptors in rat brain. *Nature* 291:493-495, 1981.

Herman, B.H., and Goldstein, A. Antinociception and paralysis induced by intrathecal dynorphin A. *J Pharmacol Exp Ther* 232:27-32, 1985.

Ho, W.K.K.; Wen, H.L.; and Ling, N. ß-Endorphin like immunoreactivity in the plasma of heroin addicts and normal subjects. *Neuropharmacology* 19:117-120, 1980.

Hollt, V.; Przewlocki, R.; and Herz, A. Beta-endorphin like immunoreactivity in plasma, pituitaries and hypothalamus of rats following treatment with opioids. *Life Sci* 23:1057-1066, 1978.

Hughes, J.; Smith, T.W.; Kosterlitz, H.W.; Fothergill, L.A.; Morgan, B.A.; and Morris, H.R. Identification of two related pentapeptides from the brain with potent opioid agonist activity. *Nature* 258:577-579, 1975.

Hung, C.Y.; Lefkowitz, S.S.; and Geber, W.F. Interferon inhibition by narcotic analgesic. *Proc Soc Exp Biol Med* 142:106-111, 1973.

Jacquet, Y.F. NMDA receptor: Central role in pain inhibition in rat periaqueductal gray. *Eur J Pharmacol* 154:271-276,1988.

Jurna, I., and Zetler, G. Antinociceptive effects of centrally administered caerulein and cholecystokinin octapeptide (CCK-8). *Eur J Pharmacol* 73:323-331, 1981.

Karkalas, J., and Lal, H. A comparison of haloperidol with methadone in blocking heroin withdrawal symptoms. *Int Pharmacopsychiatr* 8:248-251, 1973.

Kellstein, D.E., and Mayer, D.J. Spinal co-administration of cholecystokinin antagonists with morphine prevents the development of opioid tolerance. *Pain* 47:221-229, 1991.

Kest, B.; Marek, P.; and Liebeskind, J.C. The specific N-methyl-aspartate (NMDA) receptor antagonist MK-801 blocks U-50,488H, but not morphine antinociception. *Bruin Res* 589:139-142, 1992.

Knowles, R.; Palacios, M.; Palmer, R.M.J.; and Moncada, S. Formation of nitric oxide from L-arginine in the central nervous system: A transduction mechanism for stimulation of the soluble guanylate cyclase. *Proc Natl Acad Sci U S A* 86:5159-5162, 1989.

Kolesnikov, Y.A.; Pick, C.G.; Ciszewska, G.; and Pastemak, G.W. Blockade of tolerance to morphine but not to κ-opioids by a nitric oxide synthase inhibitor. *Proc Natl Acad Sci U S A* 90:5162-5 166, 1993.

Kolesnikov, Y.A.; Pick, C.G.; and Pastemak, G.W. N^G-nitro-L-arginine prevents morphine tolerance. *Eur J Pharmacol* 221:399-400, 1992.

Kovacs, G.L.; Szontagh, L.; Balaspiri, L.; Hodi, K.; Bohus, P.; and Telegdy, G. On the mode of action of an oxytocin derivative (Z-Pro-D-Leu) on morphine dependence in mice, *Neurophamacology* 20:647-651, 1981.

Krivoy, W.A.; Zimmerman, E.; and Lande, S. Facilitation of development of resistance to morphine analgesia by desglycinamide^9-lysine vasopressin. *Proc Natl Acad Sci U S A* 71:1852-1856, 1974.

Leybin, L.; Pinsky, C.; LaBella, F.S.; Havlicek, V.; and Rezek, M. Intraventricular met-enkephalin causes unexpected lowering of pain threshold and narcotic withdrawal signs in rats. *Nature* 264:458-459, 1976.

Llorens, C.; Martres, M.P.; Baudry, M.; and Schwartz, J.C. Hypersensitivity to noradrenaline in cortex after chronic morphine: Relevance to tolerance and dependence. *Nature* 274:603-605, 1978.

Maldonado, R.; Dauge, V.; Callebert, J.; Villette, J.M.; Fournié-Zaluski, M.C.; Feger, J.; and Roques, B.P. Comparison of selective and complete inhibitors of enkephalin-degrading enzymes on morphine withdrawal syndrome. *Eur J Pharmacol* 165:199-207, 1989.

Maldonado, R.; Mico, J.A.; Valverde, O.; Saavedra, M.C.; Leonsequi, I.; and Gibert-Rahola, J. Influence of different benzodiazepines on the experimental morphine abstinence syndrome. *Psychopharmacology* 105:197-203, 1991.

Marek, P.; Ben-Eliyahu, S.; Gold, M.; and Liebeskind, J.C. Excitatory amino acid antagonists (kynurenic acid and MK-801) attenuate the development of morphine tolerance in the rat. *Brain Res* 547:77-81, 1991.

Marquis, K.L.; Piesla, M.J.; Muth, E.A.; and Boast, C.A. Effects of acute/chronic MK-801 on naloxone-precipitated jumping in morphine-dependent mice. *Sot Neurosci Abstr* 17:331, 1991,

Martin, W.R., and Jasinski, D.R. Physiological parameters of morphine dependence in man tolerance, early abstinence and protracted abstinence. *J Psychol Res* 7:9-17, 1969.

Morley, J.E.; Yamada, T.; Walsh, J.H.; Lamers, H.; Wong, H.; Shulkes, A.; Damassa, D.A.; Gordon, J.; Carlson, H.E.; and Hershman, J.M. Morphine addiction and withdrawal alters brain peptide concentrations. *Life Sci* 26:2239-2244, 1980.

Mushlin, B.E., and Cochin, J. Tolerance to morphine in the rat: Its prevention by naloxone. *Life Sci* 18:797-802, 1976.

Nathenson, G.; Golden, G.S.; and Litt, I.P. Diazepam in the management of the neonatal narcotic withdrawal syndrome. *Pediatrics* 48:523-527, 1971.

North, R.A. Opioid receptor types and membrane ion channels. *Trends Neurosci* 9:114-117, 1986.

O'Brien, C.P.; Terenius, L.; Nyberg, F.; McLellan, A.T.; and Eriksson, I. Endogenous opioids in cerebrospinal fluid of opioid-dependent subjects. *Biol Psychiatry* 24:649-662, 1988.

O'Dowd, B.F. Structures of dopamine receptors. *J Neurochem* 60:804-816, 1993.

Ohnishi, T.; Saito, K.; Matsumoto, K.; Sakuda, M.; and Inoki, R. Decrease in analgesic effect of nifedipine following chronic morphine administration. *Eur J Pharmacol* 58:173-175, 1988.

O'Neill, M.F.; Dourish, C.T.; and Iversen, S.D. Morphine-induced analgesia in the rat paw pressure test is blocked by CCK and enhanced by the CCK antagonist MK-329. *Neuropharmacology* 28:243-247, 1989.

Parolaro, D.; Patrini, G.; Giagnoni, G.; Massi, P.; Groppetti, A.; and Parenti, M. Pertussis toxin inhibits morphine analgesia and prevents opioid dependence. *Pharmacol Biochem Behav* 35:137-141, 1990.

Pinsky, C.; Dua, AK.; and LaBella, F.S. Peptidase inhibitors reduce opiate narcotic withdrawal signs, including seizure activity, in the rat. *Brain Res* 243:301-307, 1982.

Przewloci, R.; Holt, V.; Duka, Th.; Kleber; G.; Gramsch, Ch.; Haarmaan, I.; and Herz, A. Long-term morphine treatment decreases endorphin levels in rat brain and pituitary. *Bruin Res* 174:357-361, 1979.

Ramarao, P., and Bhargava, H.N. Effect of thyrotrophin releasing hormone on the development of tolerance to the analgesic and hyperthermic effects of morphine in the rat. *Neuropeptides* 15:213-217, 1990.

Ramkumar, V., and El-Fakahany, E.E. Increase in ^{3}H-nitrendipine binding sites in morphine tolerant mice. *Eur J Pharmacol* 102:371-372, 1984.

Ramkumar, V., and El-Fakahany, E.E. Prolonged morphine treatment increases rat brain dihydropyridine binding sites, possible involvement in development of morphine dependence. *Eur J Pharmacol* 146:73-83, 1988.

Rasmussen, K.; Fuller, R.W.; Stockton, M.E.; Perry, K.W.; Swinford, R.M.; and Omstein, P.L. NMDA receptor antagonists suppress behaviors but not norepinephrine turnover or locus coeruleus unit activity induced by opioid withdrawal. *Eur J Pharmacol* 197:9-16, 1991.

Rattan, A.K.; Koo, K.L.; Tejwani, G.A.; and Bhargava, H.N. The effect of morphine tolerance dependence and abstinence on immunoreactive dynorphin (1-13) levels in discrete brain regions, spinal cord, pituitary gland and peripheral tissues of the rat. *Brain Res* 584:207-212, 1992.

Redmond, D.E., and Huang, Y.H. The primate locus coeruleus and effects of clonidine on opioid withdrawal. *J Clin Psychiatry* 43:25-29, 1982.

Ruiz-Gayo, M.; Durieux, C.; Fournié-Zaluski, M.C.; and Roques, B.P. Stimulation of δ opioid receptors reduces the in vivo binding of the cholecystokinin (CCK)-B-selective agonist [^{3}H]pBC 264: Evidence for a physiological regulation of CCK-ergic system by endogenous enkephalins. *J Neurochem* 59:1805-1811, 1992.

Schwartz, J.C. Metabolism of enkephalins and the inactivating neuropeptidase concept. *Trends Neurosci* 6:45-48, 1983.

Sharma, S.K.; Klee, W.A.; and Nirenberg, M. Dual regulation of adenylate cyclase accounts for narcotic dependence and tolerance. *Proc Natl Acad Sci U S A* 72:3092-3096, 1975.

Sharma, S.K.; Klee, W.A.; and Nirenberg, M. Opioid-dependent modulation of adenylate cyclase. *Proc Natl Acad Sci U S A* 74:3365-3369, 1977.

Sugerman, A.A.; Mikstal, M.W.; and Freymuth, H.W. Comparison of prazepam and placebo in the treatment of convalescing narcotic addict. *J Clin Pharmacol* 11:383-387, 1971.

Takahashi, M.; Senda, T.J.; and Kaneto, H. Role of spinal κ-opioid receptors in the blockade of the development of antinociceptive tolerance to morphine. *Eur J Pharmacol* 200:293-297, 1991.

Takemori, A.E.; Loh, H.H.; and Lee, N.M. Suppression by dynorphin A (1-13) of the expression of opioid withdrawal and tolerance in mice. *Eur J Pharmacol* 221:223-226, 1992.

Tang, J.; Chou, J.; Iadarola, M.; Yang, H.Y.T.; and Costa, E. Proglumide prevents and curtails acute tolerance to morphine in rats. *Neuropharmacology* 23:715-718, 1984.

Tao, P.L., and Liu, C.F. Chronic morphine treatment causes down-regulation of spinal A, receptors in rats. *Eur J Pharmacol* 215:301-304, 1992.

Thorat, S.N.; Barjavel, M.; Matwyshyn, G.A.; and Bhargava, H.N. Comparative effects of N^G-monomethyl-L-arginine and MK-801 on the abstinence syndrome in morphine-dependent mice. *Brain Res* 642:153-159, 1994.

Thorat, S.N., and Bhargava, H.N. Evidence for the cross-tolerance between morphine and Δ^1-tetrahydrocannabinol in mice. *Eur J Pharmacol,* in press.

Thorat, S.N.; Reddy, P.L.; and Bhargava, H.N. Evidence for the role of nitric oxide in κ-opioid tolerance in mice. *Brain Res* 621:171-174, 1993.

Trujillo, K.A., and Akil, H. Inhibition of morphine tolerance and dependence by the NMDA receptor antagonist MK-801. *Science* 251:85-87, 1991.

Tseng, L.F.; Loh, H.H.; and Li, C.H. ß-Endorphin: Cross-tolerance to and cross-dependence on morphine. *Proc Natl Acad Sci U S A* 37:4187-4189, 1976.

Tseng, L.F.; Loh, H.H.; and Wei, E.T. Effects of clonidine on morphine withdrawal signs in the rat. *Eur J Pharmacol* 30:93-99, 1975.

Valverde, O.; Mica, J.A.; Maldonado, R.; and Gibert-Rahola, J. Changes in benzodiazepine-receptor activity modify morphine withdrawal syndrome in mice. *Drug Alcohol Depend* 30:293-300, 1992.

van Calker, D.; Muller, M.; and Hamprecht, B. Adenosine regulates via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. *J Neurochem* 33:999-1005, 1979.

van Ree, J.M., and de Wied, D. Prolyl-leucyl-glycinamide (PLG) facilitates morphine dependence. *Life Sci* 19:1331-1340, 1976.

Vaught, J.L., and Takemori, A.E. Differential effects of leucine and methionine-enkephalin on morphine-induced analgesia, acute tolerance, and dependence. *J Pharmacol Exp Ther* 208:86-90, 1979.

Volpe, A.; Facchinetti, F.; Cioni, A.; and Genazzoni, A.R. Reduction of ß-endorphin levels in the amniotic fluid of heroin addicts. *Obstet Gynecol* 68:606-609, 1986.

Ward, S.J.; Portoghese, P.S.; and Takemori, A.E. Pharmacological characterization in vivo of the novel opioid, ß-funaltrexamine. *J Pharmacol Exp Ther* 220:494-498, 1982.

Watkins, L.; Kinscheck, LB.; and Mayer, D.J. Potentiation of morphine analgesia and apparent reversal of morphine tolerance by proglumide. *Science* 224:395-396, 1984.

Wen, H.L., and Ho, W.K.K. Suppression of withdrawal symptoms by dynorphin in heroin addicts. *Eur J Pharmacol* 82:183-186, 1982.

Wen, H.L.; Ho, W.K.K.; and Wen, P.Y.C. Comparison of the effectiveness of different opioid peptides in suppressing heroin withdrawal. *Eur J Pharmacol* 100:155-162, 1984.

Wesche, D.; Holt, V.; and Herz, A. Radioimmunoassays of enkephalins: Regional distribution in rat brain after morphine treatment and hypophysectomy. *Naunyn Schmiedebergs Arch Exp Pathol Pharmakol* 301:79-82, 1977.

Yamamoto, T.; Ohno, M.; and Veki, S. A selective κ-opioid agonist, U-50,488H, blocks the development of tolerance to morphine analgesia in rats. *Eur J Pharmacol* 156:173-176, 1988.

Yano, I., and Takemori, A.E. Inhibition by naloxone of tolerance and dependence in mice treated acutely and chronically with morphine. *Res Commun Chem Pathol Pharmacol* 16:721-734, 1977.

ACKNOWLEDGMENTS

The studies described from the author's laboratory were funded by a grant (DA-02598), a contract (RFP no. 271-91-9201), and a Research Scientist Development Award (K02-DA-00130) from the National Institute on Drug Abuse. Drs. K.P. Gudehithlu, P.L. Reddy, S.N. Thorat, Mr. G.A. Matwyshyn, Mr. M. Barjavel, Ms. J.M. Diaz, and Ms. A. Canterbury are acknowledged for their assistance in the preparation of this chapter.

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Future Directions in the Pharmacological Management of Hyperalgesic and Allodynic Pain States: The NMDA Receptor

Tony L. Yaksh, Sandra R. Chaplun, and Annika B. Malmberg

INTRODUCTION

Acute activation of small, high-threshold afferent fibers typically leads to the evocation of organized escape behavior and signs of agitation. In the absence of tissue injury, the escape behavior typically is stimulus dependent, essentially abating when the stimulus is removed. Distinguishable neural substrates, however, may mediate different pain states, particularly those associated with protracted afferent input (as in arthritis or in cancer pain) or those that appear as sequelae of nerve injury (such as causalgia or reflex sympathetic dystrophy). Clinical experience has indicated that these pain states often require elevated doses of narcotic (as in the first state) or, in fact, may be comparatively refractory to opiates (as in the second state). Systematic preclinical studies have provided a large body of data suggesting that these pain states involving hyperalgesia and allodynia may be mediated in part by the action of glutamate at *spinal N*-methyl-D-Aspartate (NMDA) receptors. In light of the magnitude of the problem presented by these pain states in man, the preclinical data emphatically suggest that the therapeutic use of NMDA antagonists in man has a rational basis. Because these agents have prominent supraspinal effects on mentation when given systemically and, because the site of targeted action is the spinal cord, it is believed that an appropriate method of examining clinical efficacy of these drugs is by spinal delivery.

THE HYPERALGESIC STATE

Generation of the Hyperalgesic State in Animals and Humans

Dorsal horn neurons display a progressive facilitation in the responses (called *wind-up)* (Mendell and Wall 1965) evoked by repetitive small afferent input. Concomitantly, their peripheral receptive fields of the cell

are enhanced markedly (Owens et al. 1992; Woolf and King 1990). These electrophysiological observations, descriptive of an augmentation in neuronal output to a given afferent input, are in concert with the observation that repetitive afferent input similarly may augment the response of the animal to a given noxious stimulus (e.g., hyperalgesia). Thus, as will be discussed below, the injection of an irritant such as formalin into the paw will evoke an initial burst of activity in saphenous afferents, followed by prolonged, low-level afferent activity (Heapy et al. 1987). The accompanying behavior pattern of agitation (i.e., flinching of the injected paw) characteristically is biphasic. The first behavioral phase corresponds to the initial afferent burst, but the second phase appears exaggerated in view of the low level of afferent drive. The second phase thus appears to reflect an augmented response. Such augmented reactivity similarly has been observed in humans. It has been reported that the injection of capsaicin, a selective stimulant of C-fibers, will result in an initial intense burst of pain that gradually diminishes but is followed by a period during which the observer notes that the area of the forearm distal to the site of injection displays prominent tactile allodynia and thermal hyperalgesia. To address the origin of this exaggerated processing, local anesthetic was injected into the site where the capsaicin subsequently was delivered. This transient blockade of afferent input, for a period during which the capsaicin normally evokes pain behavior, prevented the evolution of the secondary phase of hyperalgesia (LaMotte et al. 1991; Torebjork et al. 1992). These observations suggest that in humans, as in the rat, repetitive small afferent input will result in the evolution of a facilitated state of afferent processing.

Pharmacology of the Hyperalgesic State

Current studies on the pharmacology of facilitated processing (i.e., wind-up) have led to the formulation of the model that is outlined in figure 1. The spinal delivery of opioid agonists will depress the evolution of the facilitated state of dorsal horn activity (Dickenson and Sullivan 1987*a*), presumably as a result of the agonists' ability to block release of transmitter from C-fibers (Yaksh 1993), and thereby prevent the evolution of the facilitated state. Numerous studies have focused on the role of spinal glutamate receptors of the NMDA type. The spinal delivery of several NMDA antagonists, such as ketamine, 2-amino-5phosphonovalorate, or MK-801, can block the facilitated response evoked by repetitive small afferent input (Dickenson and Sullivan 1987*b*; Haley et al. 1990; Woolf and Thompson 1991). Importantly, these agents, while effective in diminishing the facilitated component, do so without significant effect

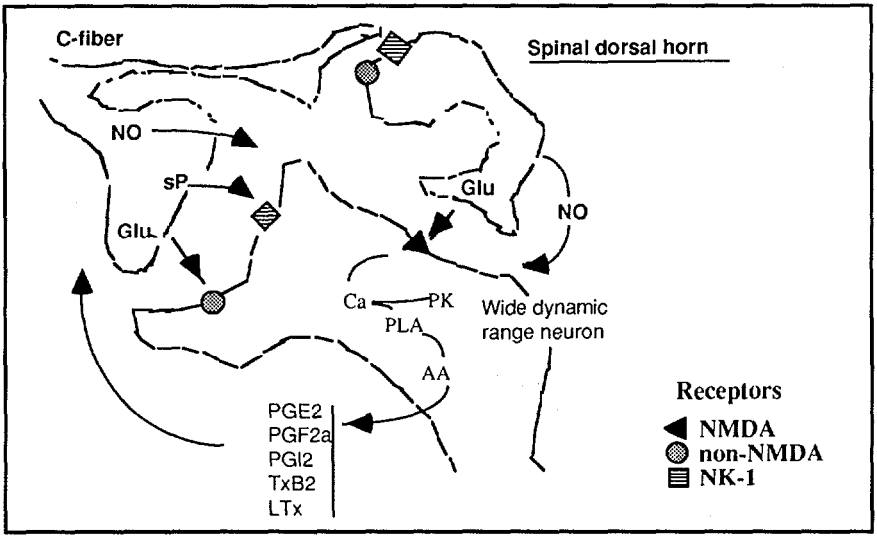

FIGURE 1. *Schematic organization of the lumbar dorsal horn presenting the organization of the neurotransmitter responses discussed in the text. C-fiber input results in the release of several neuropeptides including substance P (sP) and excitatory amino acids such as glutamate. sP and glutamate act postsynaptically on second-order neurons to evoke their direct discharge via NK-1 and non-NMDA sites. In addition, this release interacts with inter-neurons, presumably in the upper lamina of the substantia gelatinosa, to evoke the release of additional agents, including glutamate, which interact with NMDA receptors, The activation in the dorsal horn results in the increase in intracellular Ca, leading to the activation of phospholipase and the subsequent formation of arachidonic acid and the formation of several prostanoids, which move extracellularly. These prostanoids have been shown both to be directly excitatory and to increase the Ca current in the primary afferents leading to further transmitter release. In addition, this activation leads to the formation of nitric oxide (NO) by nitric oxide synthase, present in both primary afferent C-fibers and in second-order dorsal horn neurons. NO also is known to facilitate transmitter release (see Yaksh and Malmberg [1993] for references).*

This is consistent with the hypothesis that the NMDA receptor is not responsible for the primary excitation of the second-order neurons.

An important question is whether these pharmacological effects upon single-unit activity are relevant to behaviorally defined states of facilitated processing. Two models have been examined in this regard, including the formalin test and the inflamed knee joint.

Formalin Test. As noted above, following the injection of formalin into the paw of the rat (50 µl of 5 percent formalin), biphasic pain behavior appears, characterized by licking and flinching of the injected paw (Wheeler-Aceto et al. 1990) (figure 2). Given the relatively modest afferent input occurring during the second phase, it is believed that the exaggerated response during the second phase relects the evolution of a facilitated state of processing.

In rats prepared with chronic intrathecal (i.t.) catheters, the spinal delivery of several NMDA antagonists before the subcutaneous injection of formalin into the paw has been shown to produce dose-dependent suppression of phase 2 of the formalin test. Figure 3 presents the effects produced by the dose that produces maximum suppression and the maximum dose that does not produce motor dysfunction. Though not shown, the NMDA antagonists had little effect upon the phase 1 component. Moreover, as shown in figure 3, at corresponding i.t. doses, these agents in contrast to morphine were without effect upon the hot plate test.

Inflamed Knee Joint. Rats prepared 5 days previously with a lumbar it. catheter receive injection of 100 µl of a mixture of kaolin and carrageenan into the left knee joint. After 4 hours, the animal is anesthetized (1 percent halothane) and prepared with a right femoral artery catheter for blood pressure monitoring. A neonatal blood pressure cuff is wrapped loosely around the inflamed knee. Periodically, the cuff is inflated to 200 mm Hg, maintained for 120 seconds, and then deflated. In the non-inflamed knee, this compression has no effect upon either blood pressure or heart rate. However, in the inflamed knee, this treatment results in a significant increase in blood pressure (D = 22±8 mm Hg) and heart rate (D = 34±12 beats/min). The i.t. injection of morphine and several NMDA antagonists produces a significant dose-dependent reduction in the blood pressure and heart rate responses otherwise produced by the compression of the inflamed knee joint. A summary of the maximum effect produced by the i.t. drug is presented in figure 4.

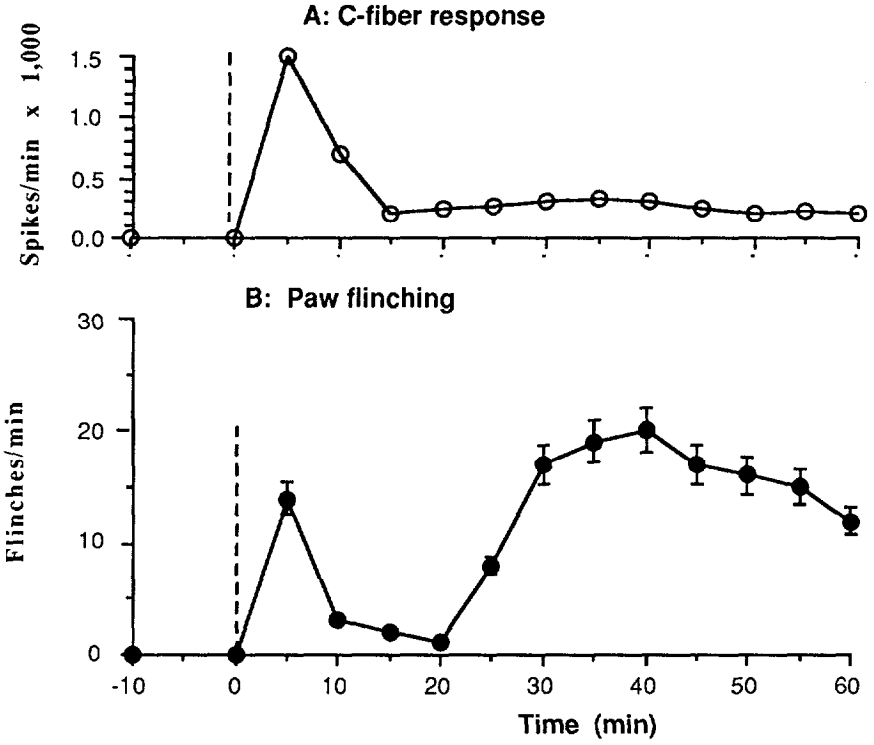

FIGURE 2. *C-fiber activity (panel a) in the saphenous nerve of the anesthetized rat and number of flinches (panel b) in the unanesthetized rat, measured after the ipsilateral subcutaneous injection of formalin into the hind paw at the time indicated by the vertical dashed line*

SOURCE: Data adapted from Heapy et al. (1987) (panel a) and Malmberg and Yaksh (1993) (panel b)

The above observations provide insight into the potential means by which certain drug treatments, such as NMDA antagonists, might diminish the contribution of secondary hyperalgesia to the postinjury pain state. Interestingly, this phenomenon may reflect a broadly applicable state. Wind-up (e.g., Dickenson and Sullivan 1987*b*) and afferent transmitter release occur in anesthetized animals (e.g., Go and Yaksh 1987). Volatile agents thus may induce a state clearly adequate to produce a loss of reported sensation and to block certain reflexes (i.e., anesthesia), but that may not be adequate to prevent the development of postinjury hyperalgesia. In recent studies, anesthetic concentrations of isoflurane

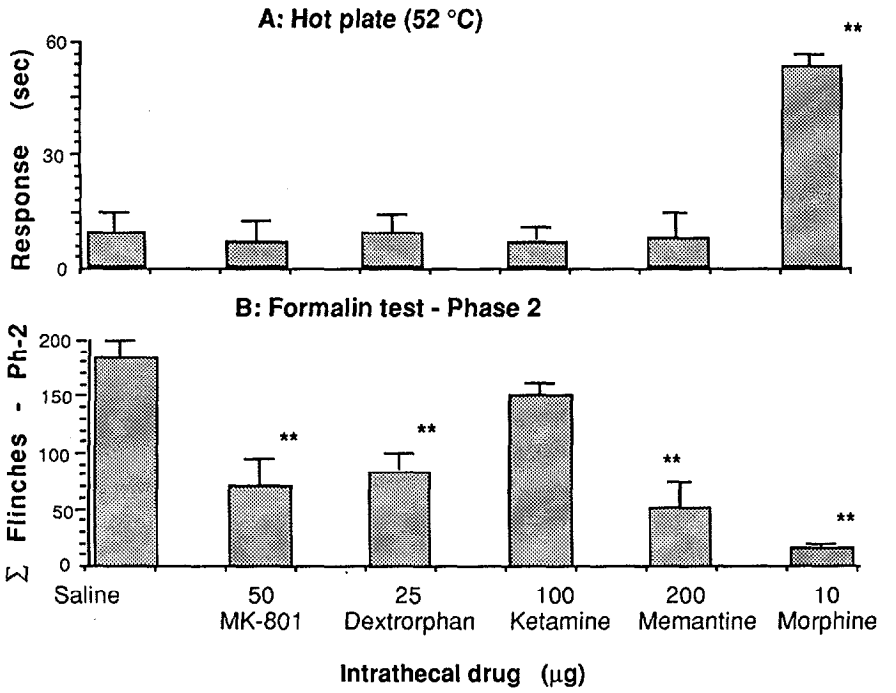

FIGURE 3. *The effects of i. t. saline, MK-801 (50 μg), dextrorphan (25 μg), ketamine (100 μg), memantine (200 μg), and morphine (10 μg) on the hot plate response latency (panel a) and the cumulative number of flinches observed during the second phase of the formalin test (panel b). Each histogram presents the mean and standard error (SE) for three to eight rats.*

KEY: ** $p < 0.01$, as compared to control

(2.3 percent) or isoflurane+N_2O applied only during phase 1 of the formalin response failed to significantly diminish phase 2 (i.e., the hyperalgesic component) of the formalin response. In contrast, the addition of morphine prior to formalin, followed by the delivery of naloxone between phases 1 and 2, resulted in a significant reduction in phase 2 response (Abram and Yaksh 1993). This argues that a hyperalgesic state may evolve in the presence of a volatile anesthetic and, furthermore, that drug treatments that reduce that evolution may serve to diminish the postoperative pain state.

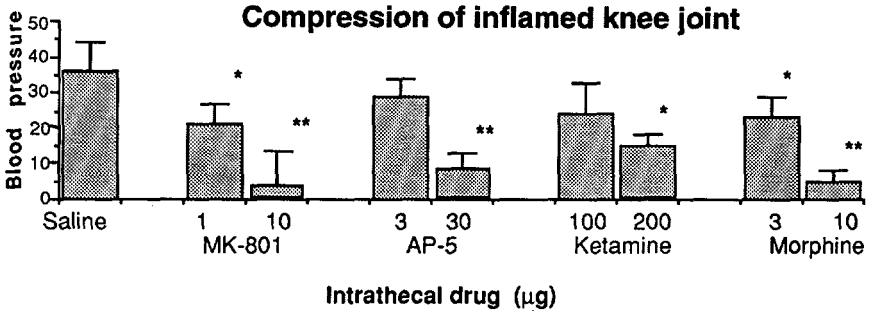

FIGURE 4. *The effects of i.t. saline, MK-801, 2-amino-Sphosphonovalorate (AP-5), ketamine, and morphine on the elevation in blood pressure produced by the compression of the inflamed knee joint in the halothane-anesthetized rat. Each histogram presents the mean and standard error for four to eight rats.*

KEY: ** $p < 0.01$ and * $p < 0.05$, as compared to control

SOURCE: Awad and Yaksh (unpublished data)

THE ALLODYNIA STATE

Generation of the Allodynic State in Animals and Humans

While the activation of high-threshold sensory afferents can evoke a well-defined pain state, it is clear that, following peripheral nerve injury, low-intensity mechanical stimuli can evoke prominent pain behavior, and this effect appears to be mediated by the activation of myelinated, low-threshold mechanoreceptors (Campbell et al. 1988). This sensory component has been documented widely to be an important component of the pain syndromes classified as causalgia and reflex sympathetic dystrophy (Bennett 1991; Burcheil and Ochoa 1991; Ochoa and Yarnitsky 1993; Payne 1986; Portenoy 1993). The syndrome of causalgia is characterized by the observation that the dysesthetic/allodynic condition may be attenuated temporarily by sympathectomy. Animal models have been developed that have permitted the systematic study of this pain state. Several such models involve compression of the nerve at the politeal fossa (i.e., sciatic nerve) (Bennett and Xie 1988; Shir and Seltzer 1990) or the tight ligation of the L5/L6 nerve roots (Kim and Chung 1992), or spinal ischemia (Hao et al. 1991; Marsala and Yaksh

1992). In these models, the application of a low-threshold tactile stimulus will evoke a reliable escape response.

The mechanisms underlying these pain states are not certain. However, in brief, several points appear to contribute:

1. Injury to the peripheral nerve results in the development of spontaneous activity in both the injured portion of the nerve (i.e., the neuroma) and in the dorsal root ganglion cells (Devor et al. 1992).

2. There is increased sympathetic innervation of large, type B ganglion cells (MacLachlan et al. 1993), which also are activated by sympathetic stimulation.

3. Myelinated afferents, which originally innervated the deeper lamina (III/IV) of the dorsal horn, now appear to sprout to innervate regions originally innervated by small, high-threshold afferents (Woolf et al. 1992). Thus, there is an anatomical basis for the possibility that large afferent input may newly evoke excitation in populations of neurons that originally were activated only by small, high-threshold afferents.

4. Following peripheral nerve lesions, there are prominent postsynaptic changes that suggest major reorganization of spinal connectivity and function. These changes include changes in dorsal horn binding (Besse et al. 1992; Garry et al. 1991); the appearance of several immediate early genes such as c-fos and c-jun (Herdegen et al. 1992*a*, 1992*b*; Molander et al. 1992); changes in the levels of messenger RNA-substance P (sP) (Noguchi and Ruda 1992); alterations in intracellular (Garrison et al. 1991) and cell surface markers (Cameron et al. 1991); and the appearance of dark staining neurons in the dorsal horn (Sugimoto et al. 1989, 1990).

Pharmacology of the Allodynic State

The innervation of dorsal horn regions by myelinated fibers originally innervated by high-threshold afferents and the possible loss of dorsal horn neurons has focused attention on a hypothesis that the allodynic state reflects a miscoding of afferent input evoked by low-threshold stimuli. In this regard, the possibility of functionally altered small interneurons is of interest, given the large number of dorsal horn interueurons containing glycine and gamma amino butyric acid (GABA)

(Carlton and Hayes 1990; Todd and Sullivan 1990). Spinal delivery of GAB A and glycine antagonists can produce a powerful allodynia, though there appears to be little alteration in the animal's response to noxious thermal stimulation (Yaksh 1989). In genetic models, such as the spastic mouse, a hyperpothic syndrome appears to exist. In this model, strychnine binding in spinal cord is reduced tenfold (White and Heller 1982). Importantly, these pharmacologically induced allodynic states generated by the loss of spinal glycinergic tone can be reversed by the spinal delivery of NMDA receptor antagonists (Yaksh 1989). Such observations have led to the consideration of whether these agents might be similarly effective in the nerve injury models of allodynia and hyperalgesia.

Bennett Model. The Bennett model (Bennett and Xie 1988) employs four loose ligatures applied to the sciatic nerve between the ischial notch and the popliteal fossa. After 5 days, the animals are tested using a Hargreaves device, in which a thermal stimulus is directed at either paw through a glass surface upon which the animal stands. For spinal drug delivery, animals are prepared in advance with a chronic i.t. catheter. Data are presented as the absolute latency to withdrawal. The spinal injection of morphine, as well as several NMDA antagonists, will result in a dose-dependent elevation in the paw withdrawal latency of the paw of the lesioned nerve. As indicated in figure 5, morphine produces a significant "analgesia," with the animal showing a near-maximum elevation toward cut off (20 seconds). In contrast, NMDA antagonists, even at maximum doses, essentially elevate the threshold of the hyperalgesic paw to "normal" (i.e., have an "antihyperalgesic" action).

Chung Model. In the Chung model, firm ligations are placed around the L5 and L6 nerves distal to the dorsal root ganglia and proximal to the main body of the sciatic nerve (Kim and Chung 1992). After 3-5 days, these animals are tested for their mechanical threshold by applying von Frey hairs to the hind paw on the side of the ligated nerves. Typically, in normal or sham-operated rats, the paw threshold (i.e., stimulus intensity that evokes a withdrawal response in 50 percent of the presentations) is greater than 15 grams (cut off). After nerve ligation, animals show withdrawal thresholds of less than 2 grams (median: 95 percent; confidence interval: 1.2 grams/0.7-1.8 grams) (Chaplan et al., in press). As indicated in figure 6, at doses below those that produce any effect upon motor function, there is a significant depression of the allodynic component of the behavioral response.

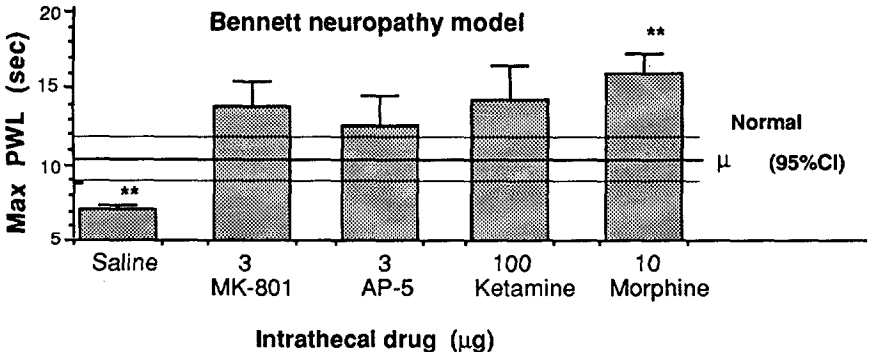

FIGURE 5. *Mean paw withdrawal latency to a thermal stimulus in the lesioned paw of rats prepared according to the Bennett and Xie (1988) model, following the i.t. injection of saline (control) and MK-801, AP-5, ketamine, and morphine (10 μg). Each histogram presents the mean and SEM of four to six rats. Control response of the nonlesioned paw (normal) is indicated by the horizontal line, with dashed lines representing 95-percent confidence interval.*

SOURCE: Data from Yamamoto and Yaksh (1991, 1992*a*)

The ability of the Chung model to separate the action of spinal opiates from that of the NMDA antagonists is an additional important element in surveying classes of drugs targeted at dysesthetic pain states that frequently are reported to be resistant (if not refractory) to the actions of opiates. The delivery of NMDA antagonists into the cerebral ventricles revealed no effect on allodynia (Chaplan and Yaksh, preliminary results). This emphasizes that the likely site of action of spinally applied NMDA antagonists is at spinal NMDA receptor sites.

CONCLUDING COMMENTS

Role of the NMDA Receptor in Therapy

As summarized in table 1, NMDA antagonists have been shown to be efficacious in a number of models of hyperalgesia and allodynia. They show little activity in models wherein the pain state is characterized by an acute afferent input.

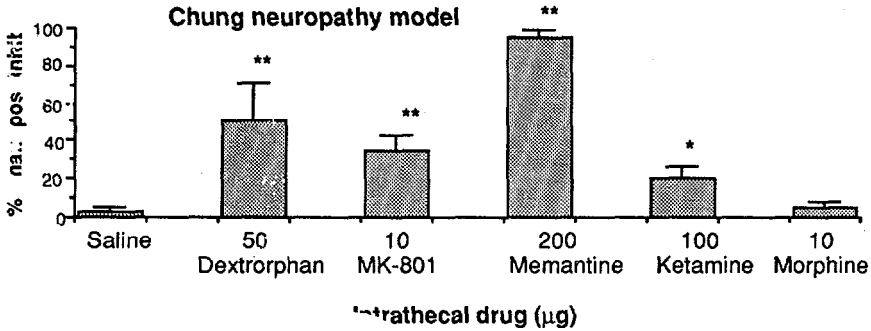

FIGURE 6. *The mean change in threshold to a mechanical stimulus in the lesioned paw of rats prepared with the Kim and Chung (1992) model of ligation following the i.t. injection of saline (control) and MK-801(10 μg) dextrorphan (50 μg) ketamine (100 μg), memantine (200 μg); or morphine (10 μg). The lesioned paws display tactile allodynia. Thus, the failure to alter the allodynia is indicated by numbers near 100 percent. Reductions in the allodynia are indicated by numbers near 0. Each histogram presents the mean and SEM of six to eight rats.*

KEY: * $p < 0.01$

SOURCE: Chaplan and Yaksh (in press)

These observations have several implications for the actions of the NMDA antagonists:

1. The NMDA receptor is responsible for the evolution of facilitated processing and, thus, agents that block NMDA receptor function (as competitive, channel-blocking, or allosteric coupling antagonists) will act as antihyperalgesics or antiallodynic agents and not as traditional analgesics.

2. Screening for development of these agents, therefore, must consider these characteristics in the models to be employed. Hyperalgesic and allodynic models will be descriptive. However, it should be stressed at this time that it is not certain that all states of allodynia are the same. Thus, the Bennett model is opioid sensitive while the Chung model is not. This observation may have clinical relevance.

TABLE 1. *Summary of antinociceptive activity of spinal NMDA antagonists in rat*

Test	IT NMDA antagonist	IT morphine	Representative references
Hot plate (52.5 °C)	0	+++	Yaksh (1989)
Formalin, phase 2	+++	+++	Coderre et al. (1992); Yamamoto and Yaksh (1992*b*)
Arthritic knee joint compression	+++	+++	Awad and Yaksh (unpublished data)
Spinal NMDA (therm hyperalg)	+++	0/+	Malmberg and Yaksh (1992)
Spinal NMDA (tactile allodynia)	+++	0	Chaplan et al. (in press)
Bennett model (therm hyperalg)	+++	++	Mao et al. (1993); Tal and Bennett (1993); Yamamoto and Yaksh (1991, 1992*a*)
Spinal strychnine	+++	0	Yaksh (1989)
Chung model (tactile allodynia)	+++	0	Chaplan et al. (in press)
Spinal ischem (tactile allodynia)	+++	+	Marsala and Yaksh (1992)
Focal spinal ischem (tactile allodynia)	+++	+	Hao et al. (1991)

KEY: 0 = No effect; + = mild blockade; ++ = moderate activity; +++ = complete dose-dependent block of pain behavior

3. The preclinical data and the hypothetical mechanism of action suggest that specific characteristics of the clinical pain state may be sensitive to the action of these classes of drugs. In this regard, based on the above commentary, it appears likely that these states of secondary hyperalgesia and hyperesthesia constitute important components of many postinjury pain states in humans and that the management of clinical pain must reflect the properties governing the origin and maintenance of such states of facilitated processing. Models of hyperalgesia often are opioid sensitive, but several forms of allodynia are not. Though controversial, this differential activity has been reported in pain patients suffering from a dysesthetic component, as occurs following nerve trauma (Arnér and Meyerson 1988; Bennett 1991) or in late stage cancer (secondary to tumor compression, radiation, or chemotherapy) (Kelly and Payne 1991), and has been argued to account in part for the reduced efficacy, or outright failure, of opiates in managing such chronic pain patients (Arnér) and Meyerson 1988; Portenoy 1993; Samuelsson and Hedner 1991).

While the mechanisms for the several processes clearly must differ, the spinal NMDA receptor appears to play an important role in diverse pain states wherein there exists an exaggerated response to small afferent or large afferent input. The homology of the preclinical models with the clinical states suggests that this class of drugs might prove to be generally applicable to several classes of pain patients. One case report has appeared in which the NMDA antagonist 3-(2-carboxypiperazin-4-yl)-propyl-1-phophonic acid was given to a single patient suffering from a dysesthetic pain state (Kristensen et al. 1992). Modest improvement was reported.

Spinal Delivery of NMDA Antagonists

An important issue is the use of the spinal route for NMDA antagonist delivery. NMDA antagonists have deleterious effects on cognition and perception through their supraspinal action (Wozniak et al, 1990). The present work emphasizes the spinal cord as the site of drug action in blocking the facilitated component of afferent processing. Consequently, the therapeutic ratio of these agents may be enhanced by the use of the spinal route of delivery. This approach has proven useful for the long-term management of pain with opiates (Driessen et al. 1989; Onofrio and Yaksh 1990; Sjoberg et al. 1991) and alpha 2 agonists (DuPen et al.

1993; Eisenach et al. 1989) and for the management of spasticity with baclofen (Coffey et al. 1993; McLean 1993).

Based on the above information, an important advance in the development of therapy for these diverse pain states will be the comprehensive characterization of the action of spinally delivered NMDA antagonists. Because of the lack of definitive data demonstrating the efficacy of these agents in these several pain states, there appear to be no industrial efforts focused on delineating the toxicity of appropriate agents. Three agents have been identified that: (1) are antagonists for the NMDA receptor; (2) are freely soluble in saline; and (3) are available for purchase as pharmaceutical grade products. These three agents are ketamine, dextrorphan, and memantine. Further studies are required to define their spinal safety.

REFERENCES

Abram, S.E., and Yaksh T.L. Morphine, but not inhalation anesthesia, blocks post-injury facilitation: The role of preemptive suppression of afferent transmission. *Anesthesiology* 78:713-721, 1993.

Arnér, S., and Meyerson, B.A. Lack of analgesic effect of opioids on neuropathic and idiopathic form of pain. *Pain* 33:11-23, 1988.

Bennett, G.J. Evidence from animal models on the pathogenesis of peripheral neuropathy and its relevance for pharmacotherapy. In: Basbaum, A.I., and Besson, J.M., eds. *Towards a New Pharmacotherapy of Pain: Beyond Morphine.* New York: Wiley, 1991. pp. 365-380.

Bennett, G.J., and Xie, Y.K. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain* 33:87-107, 1988.

Besse, D.; Lombard, M.C.; Perrot, S.; and Besson, J.M. Regulation of opioid binding sites in the superficial dorsal horn of the rat spinal cord following loose ligation of the sciatic nerve: Comparison with sciatic nerve section and lumbar dorsal rhizotomy. *Neuroscience* 50:921-933, 1992.

Burchiel, K.J., and Ochoa, J.L. Pathophysiology of injured axons. *Neurosurg Clin North Am* 2:105-1 16, 1991.

Cameron, A. A.; Cliffer, K.D.; Dougherty, P.M.; Willis, W.D.; and Carlton, S.M. Changes in lectin, GAP-43 and neuropeptide staining in the rat superficial dorsal horn following experimental peripheral neuropathy. *Neurosci Lett* 131: 249-252, 1991.

Campbell, J.N.; Raja, S.N.; Meyer, R.A.; and Mackinnon, S.E. Myelinated afferents signal the hyperalgesia associated with nerve injury. *Pain* 32:89-94, 1988.

Carlton, S.M., and Hayes, E.S. Light microscopic and ultrastructural analysis of GABA immunoreactive profiles in the monkey spinal cord. *J Comp Neural* 300:162-182, 1990.

Chaplan, S.R.; Bach, F.W.; Chung, J.M.; and Yaksh, T.L. Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Meth,* in press.

Coderre, T.J., and Melzack, R. The contribution of excitatory amino acids to central sensitization and persistent nociception after formalin-induced tissue injury. *J Neurosci* 12:3665-3670, 1992.

Coffey, J.R.; Cahill, D.; Steers, W.; Park, T.S.; Ordia, J.; Meythaler, J.; Herman, R.; Shetter, A.G.; Levy, R.; and Gill, B. Intrathecal baclofen for intractable spasticty of spinal origin: Results of a long-term multicenter study. *J Neurosurg* 78:226-232, 1993.

Devor, M.; Wall, P.D.; and Catalan, N. Systemic lidocaine silences ectopic neuroma and DRG discharge without blocking nerve conduction. *Pain* 48:261-268, 1992.

Dickenson, A.H., and Sullivan, A.F. Evidence for a role of the NMDA receptor in the frequency dependent potentation of deep rat dorsal horn nociceptive neurones following C fibre stimulation. *Neuropharmacology* 26:1235-1238, 1987*b*.

Dickenson, A.H., and Sullivan, A.F. Subcutaneous formalin-induced activity of dorsal horn neurones in rat: Differential response to an intrathecal opiate administered pre or post formalin. *Pain* 30:349-360, 1987*a*.

Driessen, J.J.; de Mulder, P.H.; Claessen, J.J.; van Diejen, D.; and Wobbes, T. Epidural administration of morphine for control of cancer pain: Long-term efficacy and complications. *Clin J Pain* 5:217-222, 1989.

DuPen, S.; Eisenach, J.C.; Alin, D.; and Zacarro, D. Epidural clonidine for intractable cancer pain. Supplement. *Reg Anesth* 18:23, 1993.

Eisenach, J.C.; Rauck, R.L.; Buzzanell, C.; and Lysak, S.Z. Epidural clonidine analgesia for intractable cancer pain: Phase I. *Anesthesiology* 71:647-652, 1989.

Garrison, C.J.; Dougherty, P.M.; Kajander, K.C.; and Carlton, S.M. Staining of glial fibrillary acidic protein (GFAP) in lumbar spinal cord increases following a sciatic nerve constriction injury. *Brain Res* 565:1-7, 1991.

Garry, M.G.; Kajander, K.C.; Bennett, G.J.; and Seybold, V.S. Quantitative autoradiographic analysis of [^{125}I]-human CGRP binding sites in the dorsal horn of rat following chronic constriction injury or dorsal rhizotomy. *Peptides* 12:1365-1373, 1991.

Go, V.L.W., and Yaksh, T.L. Release of substance P from the cat spinal cord. *J Physiol* 391:141-167, 1987.

Haley, J.E.; Sullivan, A.F.; and Dickenson, A.H. Evidence for spinal N-methyl-D-aspartate receptor involvement in prolonged chemical nociception in the rat. *Brain Res* 518:218-26, 1990.

Hao, J.X.; Xu, X.J.; Aldskogius, H.; Seiger, A.; and Wiesenfeld-Hallin, Z. Allodynia-like effects in rat after ischaemic spinal cord injury photochemically induced by laser irradiation. *Pain* 45:175-185, 1991.

Heapy, C.G.; Jamieson, A.; and Russell, N.J.W. Afferent C fiber and A-delta fiber activity in models of inflammation. *Br J Pharnzacol 90:* 164P, 1987.

Herdegen, T.; Fiallos-Estrada, C.; Schmid, W.; Bravo, R.; and Zimmermann, M. The transcription factor CREB, but not immediate-early gene encoded proteins, is expressed in activated microglia of lumbar spinal cord following sciatic nerve transection in the rat. *Neurosci Lett* 142:57-61, 1992*a.*

Herdegen, T.; Fiallos-Estrada, C.E.; Schmid, W.; Bravo, R.; and Zimmermann, M. The transcription factors c-JUN, JUN D and CREB, but not FOS and KROX-24, are differentially regulated in axotomized neurons following transection of rat sciatic nerve. *Brain Res Mol Brain Res* 14:155-165, 1992*b.*

Kelly, J.B., and Payne, R. Pain syndromes in the cancer patient. *Neural Clin* 9:937-953, 1991.

Kim, S.H., and Chung, J.M. An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain* 50:355-363, 1992.

Kristensen, J.D.; Svensson, B.; and Gordh, T., Jr. The NMDA-receptor antagonist CPP abolishes neurogenic 'wind-up pain' after intrathecal administration in humans. *Pain* 51:249-253, 1992.

LaMotte, R.H.; Sham, C.N.; Simone, D.A.; and Tsai, E.F. Neurogenic hyperalgesia: Psychophysical studies of underlying mechanisms. *J Neurophysiol* 66:190-211, 1991.

Malmberg, A.B., and Yaksh, T.L. Hyperalgesia mediated by spinal glutmate and sP receptor blocked by spinal cycloxygenase inhibition. *Science* 257:1276-1279, 1992.

Mao, J.; Price, D.D.; Hayes, R.L.; Lu, J.; Mayer, D-J.; and Frenk, H. Intrathecal treatment with dextrorphan or ketamine potently reduces pain-related behaviors in a rat model of peripheral mononeuropathy. *Brain Res* 605:164-168, 1993.

Marsala, M., and Yaksh, T.L. Reversible aortic occlusion in rats: Post-reflow hyperesthesia and motor effects blocked by spinal NMDA antagonism. Supplement. *Anesthesiology* 77:A664, 1992.

McLachlan, E.M.; Janig, W.; Devor, M.; and Michaelis, M. Peripheral Nerve injury triggers noradrenergic sprouting within the dorsal root ganglia. *Nature* 363:543-546, 1993.

McLean, B.N. Intrathecal baclofen in severe spasticity. *Br J Hosp Med* 49:262-267, 1993.

Mendell, L.M., and Wall, P.D. Response of single dorsal cord cells to peripheral cutaneous unmyelinated fibres. *Nature* 206:97-99, 1965.

Molander, C.; Hongpaisan, J.; and Grant, G. Changing pattern of c-FOS expression in spinal cord neurons after electrical stimulation of the chronically injured sciatic nerve in the rat. *Neuroscience* 50:223-236, 1992.

Noguchi, K., and Ruda, M.A. Gene regulation in an ascending nociceptive pathway: inflammation-induced increase in preprotachykinin mRNA in rat lamina I spinal projection neurons. *J Neurosci* 12:2563-2572, 1992.

Ochoa, J.L., and Yamitsky, D. Mechanical hyperalgesias in neuropathic pain patients: Dynamic and static subtypes. *Ann Neural* 33:465-472, 1993.

Onofrio, B.M., and Yaksh, T.L. Long-term pain relief produced by intrathecal morphine infusion in 53 patients. *J Neurosurg* 72:200-209, 1990.

Owens, C.M.; Zhang, D.; and Willis, W.D. Changes in the response states of primate spinothalamic tract cells caused by mechanical damage of the skin or activation of descending controls. *J Neurophysiol* 67:1509-1527, 1992.

Payne, R. Neuropathic pain syndromes, with special reference to causalgia and reflex sympathetic dystrophy. *Clin J Pain 2:59-73,* 1986.

Portenoy, R.K. Management of neuropathic pain. In: Chapman, C.R., and Foley, K.M., eds. *Current and Emerging Issues in Cancer Pain.* New York: Raven Press, 1993. pp. 351-369.

Samuelsson, H., and Hedner, T. Pain characterization in cancer patients and the analgetic response to epidural morphine. *Pain* 46:3-8, 1991.

Shir, Y., and Seltzer, Z. A-fibers mediate mechanical hyperesthesia and allodynia and C-fibers mediate thermal hyperalgesia in a new model of causalgia from pain disorders in rats. *Neurosci Lett* 115:62-67, 1990.

Sjoberg, M.; Appelgren, L.; Einarsson, S.; Hultman, E.; Linder, L.E.; Nitescu, P.; and Curelaru, I. Long-term intrathecal morphine and bupivacaine in "refractory" cancer pain: I. Results from the first series of 52 patients. *Acta Anaesthesiol Stand* 35:30-43, 1991.

Sugimoto, T.; Bennett, G.J.; and Kajander, K.C. Strychnine-enhanced transsynaptic degeneration of dorsal horn neurons in rats with an experimental painful peripheral neuropathy. *Neurosci Lett* 98:139-143, 1989.

Sugimoto, T.; Bennett, G.J.; and Kajander, K.C. Transsynaptic degeneration in the superficial dorsal horn after sciatic nerve injury: Effects of a chronic constriction injury, transection, and strychnine. *Pain* 42:205-213, 1990.

Tal, M., and Bennett, G.J. Dextrorphan relieves neuropathic heat-evoked hyperalgesia in the rat. *Neurosci Lett* 151:107-110, 1993.

Todd, A.J., and Sullivan, A.C. Light microscopic study of the coexistence of GABA-like and glycine-like immunoreactivities in the spinal cord of the rat. *J Comp Neural* 296:496-505, 1990.

Torebjork, H.E.; Lundberg, L.E.; and LaMotte, R.H. Central changes in processing of mechanoreceptive input in capsaicin-induced secondary hyperalgesia in humans. *J Physiol* 448:765-780, 1992.

Wheeler-Aceto, H.; Porreca, F.; and Cowan, A. The rat paw formalin test: Comparison of noxious agents. *Pain* 40:229-238, 1990.

White, W.F., and Heller, A.H. Glycine receptor alteration in the mutant mouse spastic. *Nature* 298:655-657, 1982.

Woolf, C.J., and King, A.E. Dynamic alterations in the cutaneous mechanoreceptive fields of dorsal horn neurons in the rat spinal cord. *J Neurosci* 10:2717-2726, 1990.

Woolf, C.J.; Shortland, P.; and Coggeshall, R.E. Peripheral nerve injury triggers central sprouting of myelinated afferents. *Nature* 355:75-78, 1992.

Woolf, C.J., and Thompson, W.N. The induction and maintenance of central sensitization is dependent on N-methyl D-aspartic acid receptor activation, implications for the treatment of post-injury pain hypersensitivity states. *Pain* 44:293-299, 1991.

Wozniak, D.F.; Olney, J.W.; Kettinger, L., III; Price, M.; and Miller, J.P. Behavioral effects of MK-801 in the rat. *Psychopharmacology* 101:47-56, 1990.

Yaksh, T.L. Behavioral and autonomic correlates of the tactile evoked allodynia produced by spinal glycine inhibition: Effects of modulatory receptor systems and excitatory amino acid antagonists. *Pain* 37:111-123, 1989.

Yaksh, T.L. The spinal actions of opioids. In: Herz, A., ed. *Handbook of Experimental Pharmacology.* Vol. 104. No. II. Berlin: Springer-Verlag, 1993. pp. 53-90.

Yaksh, T.L., and Malmberg, A.B. The central pharmacology of pain transmission. In: Wall, P.D., and Melzack, R., eds. *Textbook of Pain.* 3d ed. London: Churchill Livingstone, 1993. pp. 165-200.

Yamamoto, T., and Yaksh, T.L. Spinal pharmacology of thermal hyperesthesia induced by incomplete ligation of sciatic nerve: I. Opioid and nonopioid receptors. *Anesthesiology* 75:817-826, 1991.

Yamamoto, T., and Yaksh, T.L. Comparison of the antinociceptive effects of pre and post treatment with intrathecal morphine and MK801, an NMDA antagonist on the formalin test in the rat. *Anesthesiology* 77:757-763, 1992*b*.

Yamamoto, T., and Yaksh, T.L. Spinal pharmacology of thermal thesia induced by constriction injury of sciatic nerve: Excitatory amino acid antagonists. *Pain* 49:121-128, 1992*a*.

ACKNOWLEDGMENTS

Work cited herein was supported in part by DA-021 10 (to Tony L. Yaksh) and funds from the Reflex Sympathetic Dystrophy Syndrome Association (to Sandra R. Chaplan).

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Dual Inhibitors of Enkephalin-Degrading Enzymes (Neutral Endopeptidase 24.11 and Aminopeptidase N) as Potential New Medications in the Management of Pain and Opioid Addiction

Bernard P. Roques and Florence Noble

INTRODUCTION

It now is well accepted that the pain suppression effect of morphine is related to the interaction of this alkaloid with binding sites (μ, δ, and κ) located in the central nervous system (CNS) and, more precisely, within structures known for their involvement in the regulation of nociceptive stimuli (e.g., spinal cord, periaqueductal gray matter, and thalamus). Moreover, the wide distribution of opioid receptors in the brain probably accounts for the multiplicity of pharmacological responses (including euphoria) elicited by morphine administration. In addition to its strong analgesic potency, it should be noted that morphine displays anxiolytic and disinhibitory properties. Psychic dependence and respiratory depression, which are among the major side effects of narcotics, also could be related to overstimulation of brain receptors involved, respectively, in behavioral and bulbar respiratory control.

Therefore, there is a critical need for new analgesics able to fill the gap existing between opioid analgesics and nonnarcotic antalgics such as aspirin or paracetamol. Such new analgesics could be of major interest in a number of severe pain syndromes including postoperative pain (figure 1). Unfortunately, despite the several thousand compounds that so far have been synthesized, no potent analgesic has been found yet that possesses an analgesic potency similar to morphine or that is completely devoid of its unwanted effects. However, the discovery in the CNS of the endogenous morphine-like peptides, Met- and Leu-enkephalins (Tyr-Gly-Gly-Phe-Met and Tyr-Gly-Gly-Phe-Leu) (Hughes et al. 1975), that interact with multiple opioid receptors and are degraded by well-defined

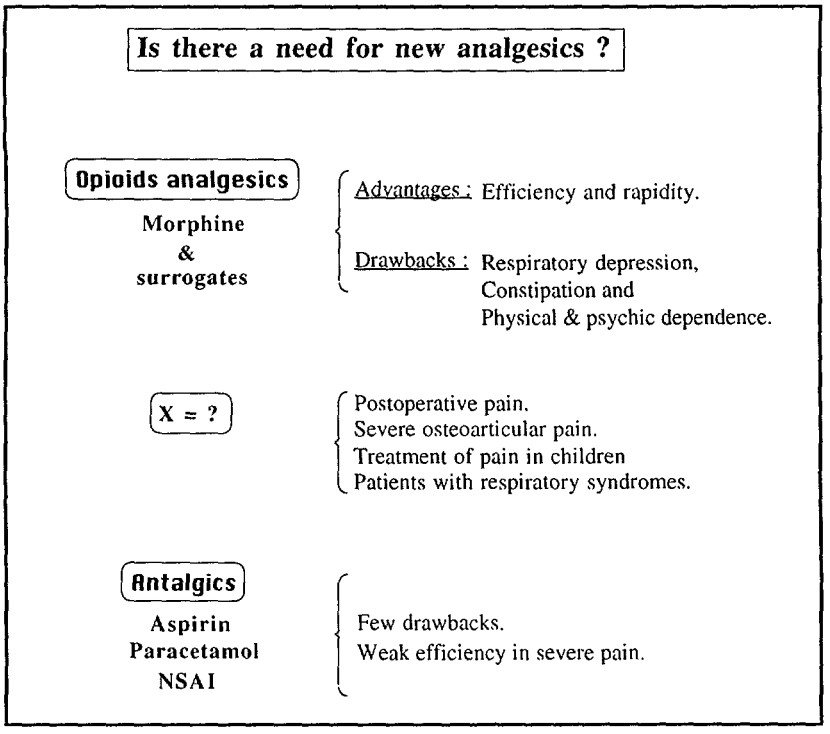


FIGURE 1. *Critical need for new analgesics able to fill the gap existing between opioid analgesics and antalgics*

metabolic pathways (Roques and Fourné-Zaluski 1987) could help to resolve, or at least to minimize, the challenging problem of opiate addiction.

In contrast to the amine and amino acid transmitters, which essentially are cleared from the extracellular space by reuptake mechanisms, the message conveyed by neuropeptides appears to be interrupted by peptidases that cleave the biologically active peptide into inactive fragments. This clearly was demonstrated for the enkephalins with the characterization of a discretely distributed "enkephalinase" activity (Malfroy et al. 1978) identical to neutral endopeptidase 24.11 (NEP) in brain regions enriched in opioid receptors and enkephalins (Waksman et al. 1986) and by the demonstration of naloxone-reversible antinociceptive responses elicited by inhibitors of this enzyme, such as thiorphan (Roques et al. 1980). Aminopeptidases, especially aminopeptidase N (APN), also have been shown to be critically involved in enkephalin inactivation (Giros et al. 1986; Hambrook et al. 1976; Waksman et al. 1985).

One of the simplest strategies for potentially useful manipulations of the endogenous opioid systems is to protect the endogenous opioid peptides from enzymatic degradation with inhibitors able to cross the blood-brain barrier (BBB). Such compounds produce their physiological effects by increasing the extracellular levels of endogenous opioid peptides released either tonically or following stimuli-evoked depolarization. Under these conditions, the effects of the inhibitors will depend upon the magnitude and duration of the enkephalin release evoked by a particular stimulus, which probably varies in the different enkephalinergic pathways, and the efficiency of metabolizing enzyme inhibition (i.e., selective inhibition of NEP or APN, or inhibition of both). These inhibitors behave, therefore, as novel analgesics, acting through a more physiological mechanism of action than morphine. It is expected that increasing the levels of endogenous opioid peptides would avoid serious drawbacks, inasmuch as they appear related to a ubiquitous overstimulation of brain opioid receptors. Moreover, since enkephalins are thought to be involved in emotional and behavioral control, enkephalin-degrading enzyme inhibitors also could behave as new psychoactive agents (Roques et al. 1985).

A second important problem is opioid addiction (i.e., psychological dependence), which is characterized by a continued craving for a drug and manifested as compulsive drug-seeking activity in the user. Many approaches have been developed to help addicts stop their drug use, but these methods do not work equally well for each type of addict, and relapse often is inevitable. It has been suggested that opioid dependence is due to or leads to a disfunctioning of the endogenous opioid system, or both (review in O'Brien 1993). Therapeutic maneuvers to increase endogenous opioid levels, such as electrostimulation or acupuncture, have been proposed in the treatment of addiction. Nevertheless, although it now is well established that such stimuli indeed increase the levels of endogenous opioid peptides, there is not yet clear evidence that they constitute successful treatments in opioid addiction. This could be related to the insufficient increase in extracellular levels of endogenous opioid peptides induced by these techniques. This limitation could be overcome with the use of mixed enkephalin-degrading enzyme inhibitors able to completely block enkephalin metabolism (Bourgoin et al. 1986; Four-nib Zaluski et al. 1984*a;* Waksman et al. 1985).

This chapter briefly and critically will review the experimental pharmacology of such inhibitors, as well as demonstrate how these

molecules could be used in therapeutics for the management of both pain and opioid addiction.

METABOLISM OF ENDOGENOUS ENKEPHALINS

Early studies on the enkephalins showed that they have a very short half-life in both in vivo and in vitro preparations. A weak and transient analgesia is obtained only for high doses (i.e., 100 µg per mouse) of intracerebroventricularly (i.c.v.) administered Met-enkephalin or Leu-enkephalin. In line with their neurotransmitter role, they are metabolized rapidly by cleavage of the Gly^3-Phe^4 bond by a peptidase, originally designated "enkephalinase" but since shown to be identical to NEP (E.C. 3.4.24.11), previously isolated from rabbit kidney (Kerr and Kenny 1974), and at the Tyr^1-Gly^2 bond level by APN (E.C. 3.4.11.2).

Interestingly, these two membrane-bound enzymes belong to the superfamily of Zn metallopeptidases, which form a large group of enzymes including the bacterial endopeptidase thermolysin (TLN) and angiotensin-converting enzyme (ACE). Such Zn metallopeptidases contain a consensus sequence of VxxHExxH (Roques 1993; Turner 1993). As shown from the crystallographic analysis of TLN, all Zn metallopeptidases have similarities in their active sites and in their respective mechanisms of action (Matthews 1988). Crystallographic studies of TLN complexed with carboxyl or with hydroxamate inhibitors have suggested that hydrolysis occurs through the formation of a pentacoordinated complex of the metal, including the oxygen of the scissile bond and the three Zn coordinating amino acids of the peptidase, without displacement of the water molecule that initially is bound to the Zn atom (Hangauer et al. 1984).

Although the sequence of NEP (Devault et al. 1987) shows only a weak homology with sequences of other Zn metallopeptidases, some of the most important amino acids in the active site of TLN appear to have been conserved (Benchetrit et al. 1988).

In NEP, His^{583} and His^{587} are two of the three Zn coordinating ligands, and a glutamate, $G1u^{584}$ plays a role in catalysis by polarizing a water molecule. The third Zn ligand was identified by site-directed mutagenesis as $G1u^{646}$. Replacement of this residue by valine results in a complete loss of enzyme activity and [^{3}H]HACBO-Gly (a selective NEP inhibitor) binding affinity (Le Moual et al. 1991). Site-directed

mutagenesis also has shown the presence in the NEP active site of two arginine resides: Arg^{102} and Arg^{747} (Bateman et al. 1989; Beaumont et al. 1991), which play a role in substrate binding. Thus, the ionic interaction of the C-terminal carboxyl group of a substrate with Arg^{102} probably explains the dipeptidylcarboxypeptidase activity of NEP clearly shown with enkephalins. The replacement of Arg^{102}, which probably is located at the enzyme surface, by Glu can be used to position inhibitors in the NEP active site (Gomez-Monterrey et al. 1993).

The specificity of the Zn metallopeptidases essentially is ensured by van der Waals and ionic interactions between their S_2, S_1, S'_1, and S'_2 subsites and the lateral chains of the corresponding P_2, P_1, P'_1, and P'_2, moieties of the substrate. The specificity is reinforced by several well-positioned hydrogen bonds occurring between the bound molecule and the polar residues of the peptidases. NEP has a broad selectivity, and its active site, especially the catalytic site, was shown to be large (Fournié-Zaluski et al. 1983), accounting for the ability of the enzyme to cut the Cys^7-Phe^8 bond of atrial natriuretic peptide (ANP) (SLRRSSCFGGRMDRIGA-OSGLGCNSFRY) (review in Stephenson and Kenny 1987). The enzyme also behaves as a very efficient endopeptidase, cleaving various linear peptides in vitro (review in Roques et al. 1993). Beside the enkephalins and Met-enkephalin-Arg-Phe, NEP shows little activity toward other opioid peptides. This is an important observation because it indicates that the "opioid" pharmacological effects induced by NEP inhibitors, and probably by mixed APN/NEP inhibitors, are due mainly to the protection of enkephalins.

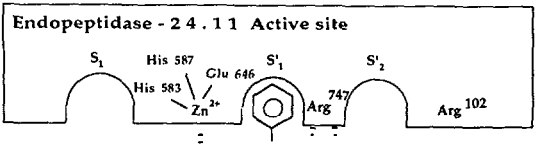
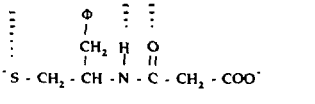
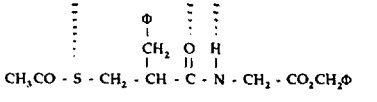
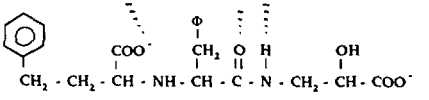
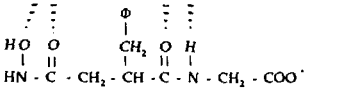
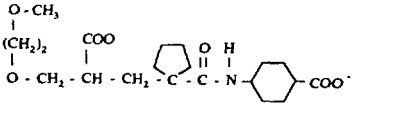
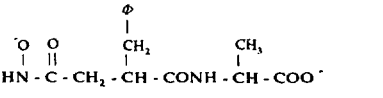
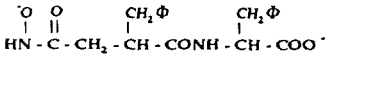
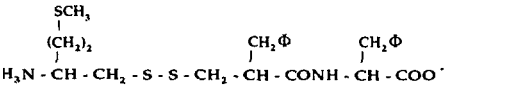
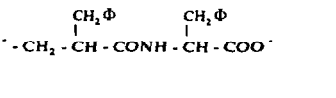
RATIONAL DESIGN OF PEPTIDASE INHIBITORS

Development of Selective NEP or APN Inhibitors

Taking into account the similarity in the active-site structures of the metallopeptidases, several series of either NEP-selective inhibitors or mixed NEP/APN inhibitors have been rationally designed (table 1).

The specificity of NEP essentially is ensured by the S'_1, subsite, which interacts preferentially with aromatic or large hydrophobic moieties, whereas the S'_2 subsite has a poor specificity (Fournié-Zaluski et al. 1979, 1981, 1984*b*; Llorens et al. 1980). These observations were used to design thiorphan (Roques et al. 1980) and retrothiorphan (Roques et al. 1983), which were the first potent synthetic NEP inhibitors described, the

TABLE 1. *Selective NEP inhibitors and mixed inhibitors NEP/APN*

		K_i (nM)	
		NEP	ACE
	*Thiorphan*	S 4 R 4	S 140 R 860
	*Retrothiorphan*	S 210 R 2.3	> 10.000
	*Acetorphan (R,S)* *Sinorphan (S)* *Retorphan (R)* (prodrugs)	> 10.000	N.D.
	*SCH 39,370*	11	> 10.000
	*HACBO-Gly*	1.7	> 10.000
	*UK 69578*	28	> 10.000
		NEP	APN
	*Kelatorphan*	1.8	380
	*RB 38A*	0.9	120
	*RB 101* (prodrug)	> 10.000	> 10.000
	*PC 18*	> 10.000	8
	*ST43*	1.5	>10.000

latter being totally unable to recognize ACE, which is involved in the control of blood pressure. Protection of the thiol and carboxyl groups of thiorphan led to acetorphan, a compound able to cross the BBB after systemic administration. In addition to acetorphan, other active NEP inhibitors containing (1) a thiol group, such as SQ 29,072 [HS-CH_2-$CH(CH_2\Phi)$-CONH-$(CH_2)_6$-COOH] (Seymour et al. 1989) or RU 44,004 [(R,S)HS-CH_2-$CH(CH_2\Phi)$-CONH-NC,H,O]; (2) a carboxyl group such as SCH 39,370 [Φ-CH_2-CH_2-CH(COOH)-NH-$CH(CH_2\Phi)$-CONH-CH_2 CH(OH)-COOH] (Sybertz et al. 1990) or UK 69,758, candoxatril [CH_3O-CH_2-CH_2-OCH_2-CH(COOH)-CH_2-X-CONH-Y, where X is cyclopentyl and Y, *p*-carbonyl cyclohexyl] (Northridge et al. 1989); and (3) hydroxamate in bidentate inhibitors such as HACBO-Gly [N-[(2R,S)-4-(hydroxy amino)- 1,4-dioxo-2(phenylmethyl)-butyl]-glycine] (Fournié-Zaluski et al. 1985; Xie et al. 1989*a*, 1989*b*) have been developed. The replacement of Gly in retro-HACBO-Gly by a highly hydrophobic aromatic moiety led to the inhibitor RB 104 [2-[(3-iodo-4-hydroxy)-phenylmethyl]-4-N-[3-hydroxyamino-3-oxo-1-(phenylmethyl) propyl]amino-4-oxobutanoic acid]. [^{125}I]RB 104 is the most potent NEP inhibitor that has been described thus far (K_I = 0.03nM), a property that has been used to directly visualize NEP in crude membrane fractions after gel electrophoresis (Fournié-Zaluski et al. 1992*a*). Another interesting series of inhibitors are the phosphorus-containing dipeptides (Elliot et al. 1985), among which is the natural competitive inhibitor of NEP, phosphoramidon.

Various natural aminopeptidase inhibitors have been isolated. These include puromycin, bestatin, amastatin, and derivatives. However, these molecules have little selectivity for APN. Simple molecules that recognize only the S_1 subsite and interact with the Zn atom were found to be highly potent APN inhibitors (Chan 1983; review in Roques et al. 1993). The bioavailability of phenylalanine-thiol (K_1 = 20 nM) was improved by introducing a hydrophobic carbamate group on the thiol function. This APN inhibitor, carbaphethiol, was reported to elicit antinociceptive activity after systemic injection in mice (Gros et al. 1988).

Development of Mixed Inhibitors of NEP and APN

The concept of mixed inhibitors was developed to take into account the previously mentioned inactivation of endogenous enkephalins by two peptidases, NEP and APN (Fournié-Zaluski et al. 1984*a*). This was achieved using the hydroxamate group as Zn-chelating moiety, hypothesizing that the strength of its coordination to the metal should

counterbalance a less-than-perfect fit of the inhibitor side chains to respective subsites of the two different enzymes (Bouboutou et al. 1984; Fournié-Zaluski et al. 1985). This indeed was obtained with bidentate-containing inhibitors such as kelatorphan and RB 38A (Fournié-Zaluski et al. 1984*a*; Schmidt et al. 1991). However, their high water solubility prevents them from crossing the BBB, and efforts to improve their bioavailability have met with little success. Therefore, new lipophilic, systemically active prodrugs such as RB 101 were developed (table 1). In these compounds, highly potent and hydrophobic thiol-containing APN and NEP inhibitors were linked by a disulphide bond. One of the main advantages of these mixed inhibitors is the relative stability of the disulphide bond in plasma, contrasting with its rapid breakdown in brain, generating the APN and the NEP inhibitors endowed with potencies in the nanomolar range toward their respective target enzymes (Fournié-Zaluski et al. 1992*b*).

ANTINOCICEPTIVE ACTIVITY OF SELECTIVE AND COMPLETE INHIBITORS OF ENKEPHALIN-DEGRADING ENZYMES

Analgesic Responses Observed After Central or Systemic Administration of Enkephalin-Degrading Enzyme Inhibitors

NEP and APN inhibitors are able to potentiate the analgesic effects of exogenous enkephalins and, more interesting, they possess a weak but significant intrinsic opioidergic action after i.c.v. injection. This has been established for thiorphan or bestatin alone or in association, retrothiorphan, and phosphoryl- or carboxyl-containing inhibitors. Different antinociceptive tests have been employed (e.g., hot plate test, tail flick test, and writhing test) and different routes of administration also have been used (table 2). All the responses observed were antagonized by prior administration of naloxone, supporting the occurrence of tonic and phasic activity of the enkephalinergic system in areas involved in the control of nociception (review in Roques et al. 1993).

However, due to the complementary role of NEP and APN in enkephalin inactivation, selective inhibition of only one of these peptidases, as shown with i.c.v. thiorphan or intravenous (i.v.) acetorphan, results in only weak antinociceptive effects (Roques et al. 1980). As expected, effects of RB 38A were compared with those induced by morphine (both

TABLE 2. *Supraspinal antinociceptive effects of selective or mixed inhibitors*

Compounds	Administration	Analgesic tests	Effects	Receptors involved	References
NEP inhibitors :					
Thiorphan	i.c.v.	Hot plate (mice)	+		*Roques et al. 1980*
Acetorphan	i.v.	Hot plate (mice)	+		*Lecomte et al. 1986*
		Writhing (mice)	++		
Phosphoramidon	i.c.v.	Paw-pressure (rats)	+		*Rupreht et al. 1983*
SCH 32826	p.o.	Writhing (mice)	++		*Chipkin et al. 1988*
		Hot plate (mice)	+		
		Inflamed paw pressure (rats)	+		
SCH 32615	PAG	Hot plate (rats)	++		*Al-Rodhan et al. 1990*
		Tail flick (rats)	++		
	i.v.	Writhing (mice)	++		*Chipkin and Coffin 1991*
APN inhibitors :					
Bestatin	i.c.v.	Hot plate (mice)	+		*Chaillet et al. 1983*
		Writhing (mice)	++		
Carbaphethiol	i.v.	Hot plate (mice)	+		*Gros et al. 1988*
NEP + APN inhibitors :					
Bestatin + Thiorphan	i.c.v.	Hot plate (mice)	+++	μ	*Chaillet et al. 1984*
Mixed-inhibitors :					
Kelatorphan	i.c.v.	Hot plate (mice)	+++		*Schmidt et al. 1991*
		Writhing (mice)	+++		
		Tail flick (mice)	+		
RB38A	i.c.v.	Hot plate (mice)	++++		*Schmidt et al. 1991*
		Writhing (mice)	++++		
		Tail flick (mice)	+++		
		Tail flick (rats)	++		
		Tail electrical stimulation :			
		• motor response	++		
		• vocalization	+		
		• vocalization post discharge	+++		
		Paw pressure	++++		
RB 101	i.v.	Hot plate (mice)	++++	μ	*Noble et al. 1992a*
		Writhing (mice)	++++	μ	
		Tail flick (rats)	++	μ/δ	
		Tail electrical stimulation :			
		• motor response	++	μ/δ	
		• vocalization	+++	μ	
		• vocalization post discharge	++++	μ	
	i.p.	Hot plate (mice)	++++	μ	
	i.p.	Formalin (mice)	++		*Noble et al. submitted*

NOTE: Plus signs indicate the size of the effect of the drug used in each test. One plus sign indicates a small effect, two and three indicate medium effects, and four indicate high effects.

compounds were administered i.c.v.) on various assays commonly used to select analgesics. In all the tests, RB 38A induced naloxone-mixed inhibitors are much more effective. Thus, the antinociceptive antagonized antinociceptive responses greater than thiorphan or thiorphan plus bestatin, showing that endogenous enkephalins, completely protected from metabolizing enzymes, are able to elicit pain suppressive effects. This is demonstrated not only in assays where naloxone has been shown to produce pronociceptive effects but more generally in morphine-sensitive tests (Schmidt et al. 1991) (table 2).

However, even at very high concentrations (150 μg, i.c.v.), at which they have been shown to completely inhibit enkephalin metabolism (Bourgoin et al. 1986; Waksman et al. 1985), mixed inhibitors are unable to produce the maximum analgesic effect induced by morphine, except in the hot plate and the writhing tests (Schmidt et al. 1991). This suggests that the local increase in enkephalin concentration produced by mixed inhibitors remains too low to saturate opioid receptors, in agreement with the results of in vivo binding experiments (Meucci et al 1989; Ruiz-Gayo et al. 1992).

Unfortunately, due to their high water solubility, the bidentate-containing inhibitors are unable to cross the BBB. This precludes investigations of their effects that use a clinically relevant route of administration. As previously discussed, a new series of compounds able to cross the BBB and to inhibit NEP and APN recently was developed. RB 101 is the first systemically active prodrug generating, through a biologically dependent cleavage of the disulfide bond, the potent APN [(S)2-amino-1-mercapto-4-methylthiobutane; K_I = 11 nM] and NEP [N-[(R,S)-2-mercapto-methyl-1-oxo-3-phenylpropyl]-L-phenylalanine; K_I = 2 nM] inhibitors. RB 101 easily crosses the BBB, as shown by the complete inhibition of cerebral NEP following i.v. injection in mice (Noble et al. 1992*a*). The prodrug induces strong, dose-dependent antinociceptive responses in the hot plate (ED_{50} = 9 mg/kg, i.v.) and the writhing (ED_{50} = 3.25 mg/kg, i.v.) tests in mice and the tail flick and the tail electric stimulation tests in rats. RB 101 also was shown to be active with ED, of 80 mg/kg in the hot plate test in mice using intraperitoneal (i.p.) administration. In all of the tests used, the pain-alleviating effects of the mixed inhibitor was suppressed by naloxone (Noble et al. 1992*a*) (table 2).

Moreover, in the presence of increasing concentrations of naloxone, i.v. injection of the mixed inhibitor RB 101 or the highly u-selective agonist Tyr-D.Ala-Gly-(Me)Phe-Gly.ol (DAMGO) resulted in similar pA_2 values

(Noble et al. 1992*a*). In addition, thiorphan and acetorphan both were shown to induce analgesia in DBA/2J mice but not in C57BL/6J, a strain characterized by a genetic insensitivity to the μ-preferential agonist morphine (Michael-Titus et al. 1989). All these results support a preferential involvement of μ receptors in supraspinal analgesia, at least with respect to thermal nociceptive stimuli (Baamonde et al. 1991; Chaillet et al. 1984; Chang et al 1982; Daugé et al. 1987; Fang et al. 1986; Noble et al. 1992*a*; Shook et al. 1987).

Inhibitor-Induced Spinal Antinociception

The enkephalins are found in high levels in the spinal cord, especially in the substantia gelatinosa, a region also enriched in μ and δ opioid receptors and in NEP (Waksman et al. 1986). In contrast to the controversy regarding their supraspinal involvement in pain suppression, it seems clear that μ and δ receptors exert an independent control of nociception at the spinal cord level (Dickenson 1991).

The antinociceptive properties of kelatorphan, locally infused onto the spinal cord, are inhibited by the selective δ opioid antagonist N,N-diallyl-Tyr-Aib-Aib-Phe-Leu (Dickenson et al. 1986) and are shown to be additive with those of the p-selective agonist DAMGO, but not with those of the selective δ agonist Tyr-D.Ser. (o-tertiobutyl)-Gly-Phe-Leu-Thr (DSTBULET) (Dickenson et al. 1988), confirming that endogenous enkaphalins and Q-selective agonists act on a common binding site to produce spinal antinociception. Intrathecal (i.t.) kelatorphan also is efficient in reducing the more prolonged noxious stimulation induced by subcutaneous (s.c.) formalin (Sullivan et al. 1989).

Several studies have used the expression of immediate early genes as markers for neuronal activity in an attempt to differentiate the pain modulatory effects of exogenously administered opioids from tonically released endogenous opioid peptides. When administered i.v. before heat stimulation, both morphine and, to a lesser extent, kelatorphan and RB 101 reduce the induction of immediate early genes, such as c-fos, in the superficial dorsal horn and the deep dorsal horn of rats (Abbadie et al., submitted; Tölle et al. 1992) (figure 2). This confirms the involvement of endogenous opioid systems in the modulation of nociceptive processes at the spinal level. On the other hand, the decrease of immediate early genes expression by kelatorphan and its increase by naloxone supports the existence of a tonically active opioidergic gating system in the dorsal horn. Accordingly, thiorphan and SCH 32,615 display strong naloxone-

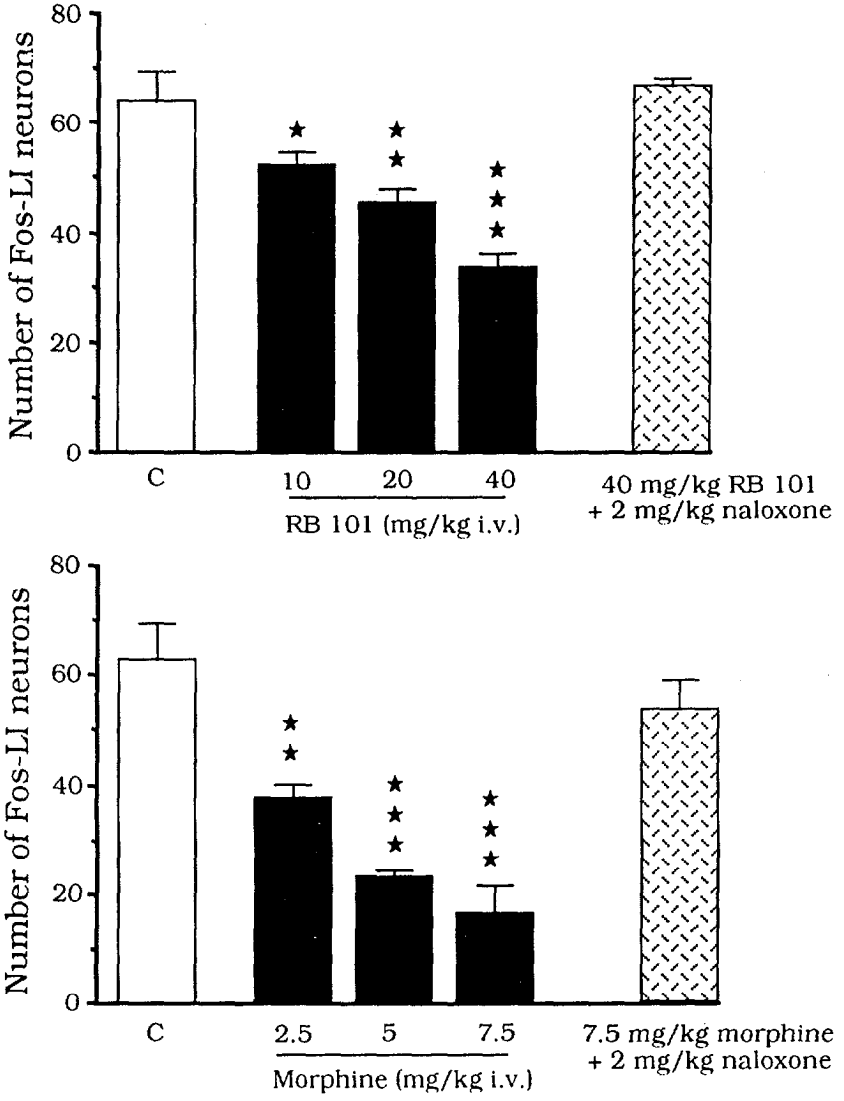


FIGURE 2. *Effects of RB 101 and morphine on Fos-like immunoreactivity (Fos-LZ) in the superficial dorsal horn and the deep dorsal horn 2 hours after heat stimulation (52°C for 15 sec) applied to the rat's right foot and reversion by naloxone (2 mg/kg, s.c.). Rats were treated 10 min prior to stimulation.*

KEY: ★|$p < 0.05$, ★★|$p < 0.01$, and ★★★$p < 0.001$, as compared to control

reversible antinociceptive responses in the hot plate, paw pressure, and tail flick tests in rats after i.t. drug administration (Oshita et al. 1990; Yaksh and Harty 1982). Moreover, electrophoretic administration of kelatorphan in the substantia gelatinosa of spinal cats leads to naloxone-reversible inhibition of nociceptive responses and marked potentiation of co-administered Met-enkephalin (Morton et al. 1987).

Given that there are pharmacologically discernable μ and δ receptor populations in the spinal cord that independently modulate noxious transmission, mixed inhibitors such as kelatorphan and selective δ agonists may be of clinical interest in patients insensitive or tolerant to morphine. These drugs also may be useful as a means of avoiding or minimizing unwanted side effects mediated by stimulation of the μ receptor. This novel approach to analgesia has provided promising preliminary clinical results after i.t. administration of either kelatorphan (Meynadier, Fournié-Zaluski, and Roques, personal communication, May 1987) in morphine-tolerant patients or bestatin and thiorphan in normal patients (Meynadier et al. 1988).

Peptidase Inhibitors in Chronic Pain

Using a centrally integrated test (i.e., the vocalization threshold to paw pressure), it has been established that the mixed inhibitor kelatorphan or PC 12, a derivative of RB 101 (Fournié-Zaluski et al. 1992*a*), when administered systemically, produces a potent antinociceptive effect. This effect is seen in normal rats and in arthritic rats used as an experimental model of clinical pain, at doses as low as 5 mg/kg, i.v. (Kayser et al. 1989; Perrot et al. 1993). These mixed inhibitors were found to be much more effective in arthritic rats than in normal ones. Given the very weak passage of kelatorphan into the brain and the lack of changes in the level of NEP or μ and δ opioid receptors in the arthritic rats (Delay-Goyet et al. 1989*a*), its strong antinociceptive effects in inflammatory pain raise the question of a possible action at the level of peripheral nociceptors, where all opioid targets including NEP seem to be present (Stein et al. 1988, 1989, 1993). Moreover, on a model of unilateral inflammatory "pain" (i.e., intraplantar injection of Freund's complete adjuvant), the stress of a forced cold water swim induced a greater elevation of paw pressure threshold in inflamed than in noninflamed paws (Stein et al. 1990).

This antinociceptive response was significantly potentiated and prolonged in rats that received an intraplantar injection of thiorphan plus bestatin, showing that this effect seems to be mediated at least in part by

endogenous opioid peptides. Evidence for a peripheral site of action of enkephalin-like peptides in this model was provided by the antagonism of the action of the inhibitors by s.c. quaternary naltrexone administered at doses shown to act exclusively at a peripheral level (Parsons and Herz 1990). As expected, slightly higher effects were observed in the same model following systemic administration of RB 38A or RB 101. Nevertheless, in this study a reduction of the RB 101-induced antinociceptive response also was observed after central administration of methylnaloxonium. This could suggest a possible action at the supraspinal level of the mixed inhibitor (Maldonado et al., in press).

The formalin test measures the response to a long-lasting nociceptive stimulus and, thus, may bear a closer resemblance to clinical pain and be analogous to human postoperative pain. I.p. injection of RB 101 (50 mg/kg) induces antinociceptive responses during early (0-5 minutes postformalin) and late (20-30 min postformalin) observation phases. Nevertheless, in contrast to the results obtained in the hot plate and tail flick tests, the antinociceptive effects of RB 101 are not potentiated by administration of selective cholecystokinin (CCK) antagonists of brain receptors (CCK,). The facilitatory effects of CCK_B antagonists are observed only on the antinociceptive responses induced by morphine during the late observation phase (Noble et al., submitted).

Morphine, DAMGO, and kelatorphan also were found to be highly active in rats with pain-related disorders as a result of peripheral mononeuropathy (Attal et al. 1991). This result emphasized that some patients with neuropathic pain may benefit from long-term treatment with mixed inhibitors and that it is necessary to perform new clinical investigations of opioid responsiveness in human painful neuropathies.

Interactions Between the Cholecystokinin and Enkephalin Systems in the Control of Pain

CCK, shown to be present in very high concentrations in the brain (Vanderhaeghen et al. 1980), interacts with nanomolar affinities with two binding sites. These are designated the CCK_A and CCK_B receptors and have been shown to be distributed discretely in brain (Moran et al. 1986). Anatomical studies have shown that enkephalins and CCK have strikingly similar distributions within many regions of the CNS. This overlapping distribution of the peptides and their respective receptors in pain-processing regions of the brain and the spinal cord (Gall et al. 1987; Pohl et al. 1990) have focused attention on the role of CCK in

nociception. Several studies have reported a naloxone-reversible antinociceptive effect of cholecystokinin octapeptide (CCK,) or its analog in several antinociceptive tests, such as the hot plate, writhing, and tail flick tests (review in Baber et al. 1989).

However, it also has been suggested that CCK_8 could act as an endogenous antiopioid peptide (Faris et al. 1983; Itoh et al. 1982). Accordingly, numerous studies have shown that peripherally administered CCK antagonists potentiate opioid antinociceptive responses, confirming a functional antagonism of the opioid system by the CCK system (Watkins et al. 1985; Wiesenfeld-Hallin et al. 1990).

Recently, the existence of regulatory mechanisms between CCK and enkephalin systems in the control of pain have been proposed. Activation of CCK_A receptors by i.c.v. administration of BDNL potentiates the analgesic effects of RB 101 and DAMGO (i.v.) while activation of CCK_B receptors by i.c.v. injection of BC 264 reduces them (Derrien et al 1993; Noble et al. 1993*a*). Taken together these results suggest the occurrence of a regulatory mechanism between CCK and enkephalin systems in the control of pain. Schematically, stimulation of CCK_A receptors would enhance opioid release (in agreement with a previous study using CCK_8 and a cocktail of peptidase inhibitors [Hill et al. 1987]) or to a direct improvement in the efficacy of transduction processes occurring at the μ sites, which might be allosterically evoked by CCK_A, site occupation (Magnuson et al. 1990). In contrast, CCK_B, receptor activation could, in turn, negatively modulate the opioidergic system (figure 3). This is supported by the blockade of CCK_B, binding sites by selective antagonists, which significantly increases the antinociceptive responses induced by RB 101 in the rat tail flick test and the mouse hot plate test (Maldonado et al. 1993). Administration of the selective CCK_B antagonist PD 134,308 at 0.3, 1, and 3 mg/kg (i.p.) potentiates very strongly the antinociceptive responses observed after injection of RB 101 (2.5,5, and 10 mg/kg, iv.). Thus, the analgesia observed after the association of PD 134,308 at doses as low as 0.3 mg/kg and 3 mg/kg with RB 101 (5 mg/kg) are about 400 percent and 800 percent higher than that observed with RB 101 given alone in the tail flick test in rats, respectively (Valverde et al., in press) (figure 4).

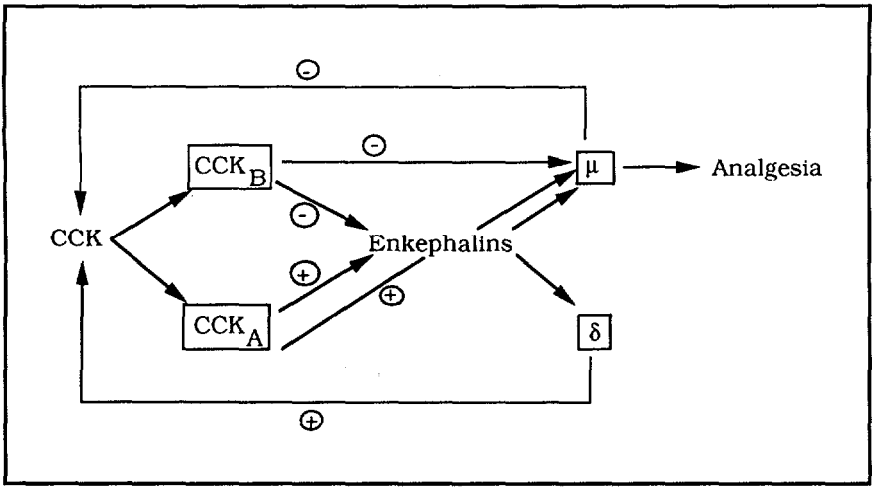


FIGURE 3. *Hypothetical model of the interactions between CCK via CCK_A and CCK_8 receptors and the opioid system via δ opioid and M opioid receptors. CCK agonists, endogenous or exogenous, stimulate the CCK_B and/or the CCK_A receptors, which can modulate the opioidergic systems either directly (via binding of opioid agonists or via C-fiber-evoked activity) or indirectly (via the release of endogenous enkephalins). In addition, activation of p opioid receptors, which leads to antinociceptive responses, could negatively modulate the release of endogenous CCK, while δ opioid receptors may enhance it.*

BEHAVIORAL EFFECTS OF PEPTIDASE INHIBITORS

Various pharmacological and biochemical studies have shown that enkephalins are involved in the control of behavior such as arousal, locomotion, self-administration, self-stimulation, learning, and memory functions through modulation of the motor (nigrostriatal) and limbic cortical (mesocorticolimbic) dopaminergic systems.

A link between opioid and dopaminergic systems has been demonstrated by 6-hydroxydopamine-induced lesions of the dopamine neurons of the ventral tegmental area (VTA) and chronic neuroleptic treatment, both of which potentiate the behavioral effects of exogenous opioids of kelatorphan infusion into the nucleus accumbens (Maldonado et al. 1990*a*; Stinus et al. 1985). Thus, dopamine receptor antagonists have been found to facilitate the behavioral effects induced by acute

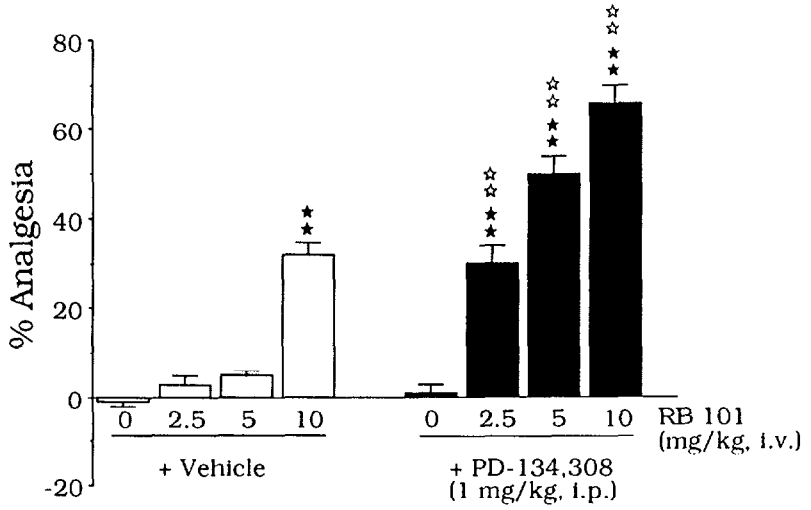


FIGURE 4. *Antinociceptive effects of RB 101 (i.v.) in the tail flick test in rats and potentiation by the selective CCK_B antagonists PD 134,408 (1 mg/kg, i.p.)*

KEY: ★★p < 0.01, as compared to control group; ☆☆]p < 0.01, as compared to the same dose of RB 101 without PD 134,308

administration of the mixed inhibitor, which was maximal 2-3 weeks after the beginning of treatment-a delay corresponding to the first appearance of the antipsychotic effects of neuroleptics (Maldonado et al. 1990*a*). This could be related to the observed disinhibition of the enkephalinergic neuron, normally negatively controlled by the dopaminergic input, with an increase in preproenkephalin expression (Hong et al. 1979; Le Moine et al. 1991; Morris et al. 1988; Scott Young et al. 1986; Thal et al. 1983). Therefore, alterations in the opioidergic system, very likely through its interrelations with the dopaminergic pathway, could take place in a neuronal system that is involved critically in the control of mood (McLennan and Maier 1983; Roques et al. 1985).

An interaction between opioidergic and dopaminergic systems also has been demonstrated by the clear antidepressant-like effects observed in the forced swimming (Porsolt) and suppression of motility tests following i.v. administration of the prodrug RB 101 in mice. Indeed, these effects, which were shown to be related to δ receptors and D_1 receptor activation,

produced an increase in dopamine turnover in the striatum (Baamonde et al. 1992) (figure 5[a]).

On the other hand, it recently has been shown that selective CCK_B antagonists induce antidepressant-like effects after systemic administration in the suppression motility and Porsolt tests, which are antagonized both by the δ-selective antagonist naltrindole and by dopamine antagonists (Derrien et al. 1994; Hernando et al., submitted) (figure 5[b]). These results indicate that CCK_B antagonists could block centrally located CCK_B receptors, thus reinforcing the antidepressant-like effects induced by δ opioid receptor stimulation. This possibly may occur through an increase of extracellular dopamine contents in some brain areas involved in depression. Moreover, recent studies have shown that the antidepressant-like effects of RB 101 could be increased by selective CCK_B antagonists in the suppression motility test in mice (Smadja et al., submitted). These data suggest a potential use of CCK_B antagonists, alone or combined with mixed enkephalin-degrading enzyme inhibitors, in the treatment of depressive syndromes.

INTEREST OF ENKEPHALIN-DEGRADING ENZYME INHIBITORS ALONE OR IN COMBINATION WITH CCK_B ANTAGONISTS IN THE TREATMENT OF OPIOID ADDICTION

In addition to serious drawbacks such as respiratory depression, the development of analgesic tolerance and physical and psychic dependence (observed during chronic administration) can limit the clinical use of morphine and surrogates. Typically, long-term administration of opiates leads to a reduction in the magnitude and duration of effects produced by a given dose and to physical dependence (defined as the appearance, upon withdrawal of the drug [or administration of an antagonist] of behavioral changes that are not observed before or during administration of the drug). Addiction or psychological dependence implies the compulsive self-administration of the drug caused by both its reinforcing or rewarding effects and the unpleasant experience (i.e., abstinence syndrome) produced by the sudden interruption of its consumption.

Tolerance, Dependence, and Side Effects of Selective and Mixed Inhibitors of NEP and APN

As mentioned above, it was hoped that agents that increase levels of endogenous opioid peptides would avoid the serious drawbacks of

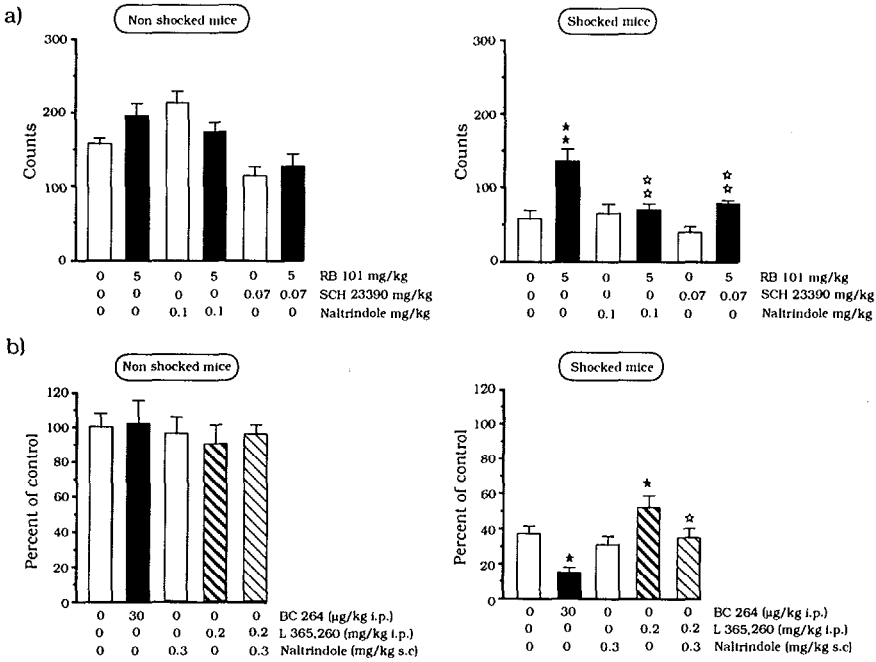


FIGURE 5. *(Panel a) Effects of naltrindole (δ antagonist) and SCH 23,390 (D_1 antagonist) on the effects induced by i.v.-injected RB 101 in the conditioned supression of motility test in mice. ★★$p < 0.01$, as compared to control; ☆☆$p < 0.01$, as compared to the same dose of RB 101 without antagonist. (Panel b) Effects of the highly selective CCK_B agonist BC 264, the selective CCKB antagonist L 365,260, and naltrindole on the antidepressant-like effects induced by L 365,260 in the conditioned suppression of motility test in mice. ★$p < 0.05$, as compared to control; ☆$p < 0.05$, as compared to the same dose of L 365,260 without naltrindole.*

morphine or surrogates, inasmuch as these drawbacks could be related to an overstimulation of tonically or phasically activated opioid receptors in all brain areas. After i.v. administration in freely moving rats at concentrations that give analgesic responses in the vocalization test, kelatorphan had no significant effect on respiratory frequency and tidal volume (Boudinot et al. 1988). Likewise, the NEP inhibitors acetorphan (Lecomte et al. 1986) and SCH 34,826 (Chipkin et al. 1988) also have been reported to be devoid of respiratory effects. Kelatorphan

ionophoretically applied into the nucleus ambigus of cats produces a low reduction of respiratory frequency (Morin-Surun et al. 1992).

Previous studies have shown that chronic i.c.v. infusion of mixed inhibitors of enkephalin-degrading enzymes such as RB 38A leads to a tolerance weaker than that evoked by selective μ or δ opioid agonists used under the same conditions (Maldonado et al. 1990*b*). Under these conditions, administration of DAMGO induced a severe withdrawal syndrome, evidenced by large weight loss and behavioral changes (i.e., jumping, teeth chattering, mastication, wet dog shakes, lacrimation, salivation, and diarrhea), while DSTBULET and RB 38A produced a moderate physical dependence. The promising results obtained with RB 38A (i.e., weak tolerance and physical dependence) were confirmed after administration by a more clinically relevant route of administration. Indeed, no signs of withdrawal were observed after administration of naloxone in animals chronically treated with RB 101 (Noble et al. 1992*b*). Moreover, chronic administration of the mixed inhibitor prodrug did not induce tolerance or cross-tolerance with morphine (Noble et al. 1992*c*) and, unlike morphine, did not induce psychic dependence (Noble et al. 1993*b*) (figure 6).

Side effects following chronic treatment with opiates probably are due to multiple cellular events. The moderate degree or the lack of these effects observed after chronic treatments with the mixed inhibitors (RB 38A or RB 101) could be explained by a weaker but more specific stimulation of the opioid binding sites by the tonically released endogenous opioids, thus minimizing receptor desensitization or down-regulation that occurs after the ubiquitous stimulation of opioid receptors by morphine (Morris and Herz 1989; review in Nestler 1992; Nestler and Tallman 1988). Moreover, it has been demonstrated that in the locus coeruleus, which is the most critical structure implicated in the development of morphine dependence (Duman et al. 1988; Maldonado et al. 1992), there is little or no tonic endogenous opioid action (Williams et al. 1987). This has been confirmed recently by Drolet and colleagues (1992), who showed that enkephalinergic inputs to locus coeruleus are not tonically active during resting conditions but may become active during stressful conditions to exert a moderating effect upon the activity of locus coeruleus neurons. Another possible reason could be weaker adenylate cyclase expression and protein phosphorylation (Matsouka et al., in press; review in Nestler 1992) or difference in the efficiency of triggering the release of endogenous antiopiate peptides.

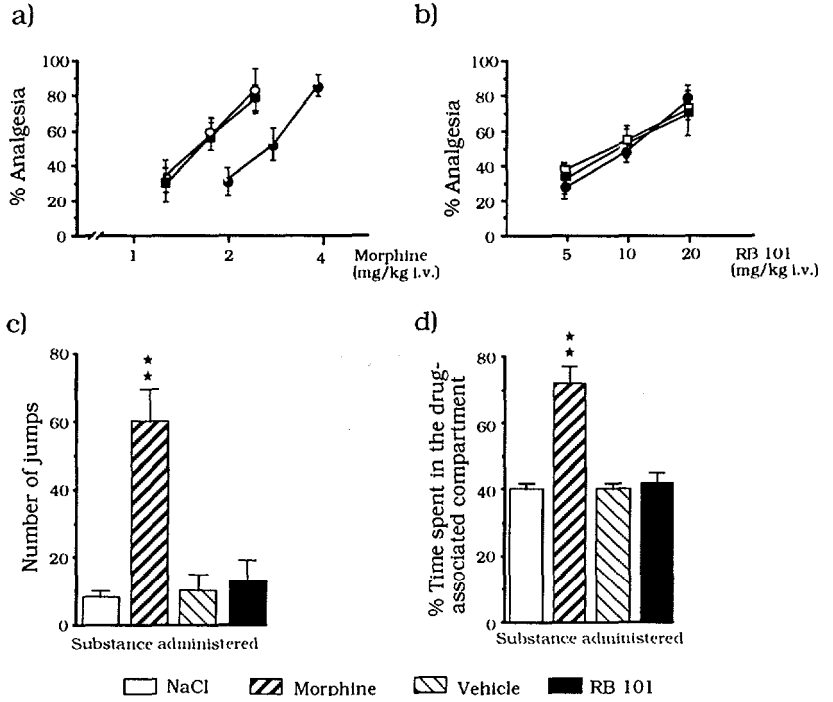


FIGURE 6. *(Panel a) Antinociceptive dose-response curves recorded in the hot plate test (i.e., jump response) IO min after i.v. administration of morphine to mice chronically pretreated with saline ○, RB 101 ■ (80 mg/kg), or morphine ● (3mg/kg) i.p. twice daily, for* 4 *days. (Panel b) Antinociceptive dose-response curves recorded in the hot plate test (jump response) 10 min after i.v. administration of RB 101 to mice chronically pretreated with vehicle □, RB 101 ■ (80 mg/kg), or morphine ● (3 mg/kg) i.p., twice daily, for* 4 *days. (Panel c) Comparison of the withdrawal symptoms induced by naloxone after chronic treatment with morphine (6 mg/kg) or RB 101 (160 mg/kg) injected i.p. twice daily for 5 days. (Panel d) Comparison of the psychic dependence induced by chronic morphine (6mg/kg) or RB 101 (160 mg/kg) injected i.p. in the place preference test.*

KEY: ★★ $p < 0.01$, as compared with other groups

On the other hand, because of their higher intrinsic efficacy (Noble and Roques, submitted; Porreca et al. 1990), enkephalins need to occupy fewer opioid receptors than morphine to give the same pharmacological responses. This also may explain the lack of or moderate side effects observed after chronic treatment with mixed inhibitors, as compared to opiates, as well as the lack of cross-tolerance observed between RB 101 and morphine.

The biochemical mechanisms that are involved in reward systems are unknown, but dopaminergic neurons that project from the VTA to the nucleus accumbens may play a major role in the euphorogenic properties of opiates (Bozarth 1986; Phillips and Le Piane 1982). The failure of RB 101 to establish a conditional place preference probably results from a lower recruitment of opioid receptors and a poorer capability to modify intracellular events for endogenous enkephalins than for morphine. This hypothesis is supported by the weaker changes in dopamine release in the nucleus accumbens after administration in the VTA of kelatorphan (figure 7), compared with a μ opioid agonist (Daugé et al. 1992), and by the apparent absence of effects on the levels of dopamine and metabolites in the nucleus accumbens following i.v. administration of acetorphan (Dourmap et al. 1990). Accordingly, when injected in the VTA of rats, kelatorphan, unlike morphine or μ agonist DAMGO, either has no effect or slightly decreases the rate of intracranial stimulation, a response typically triggered by addictive drugs (Heidbreder et al. 1992).

Clinical Interest of the Enkephalin-Degrading Enzyme Inhibitors in Opioid Addiction Treatment

It is necessary to emphasize that, in contrast to tolerance or physical dependence, addiction is not simply a biological phenomenon. Psychological, environmental, and social factors can strongly influence its development. All these factors could explain the important variability of the clinical syndrome of addiction, as well as the observation that many approaches have been developed to help addicts to stop their drug use; it is important to note that these methods do not work equally well for each type of addict. Moreover, evaluation of several aspects such as personality, background, psychiatric state, duration, extent, and type of drug use may be evaluated to increase the chance of success in the management of opioid addiction. Thus, a combination of psychotherapy and pharmacotherapy so far has been the most promising approach in treatment programs, where the most difficult aspect is the strong susceptibility to relapse. Relapse may occur long after the drug has been

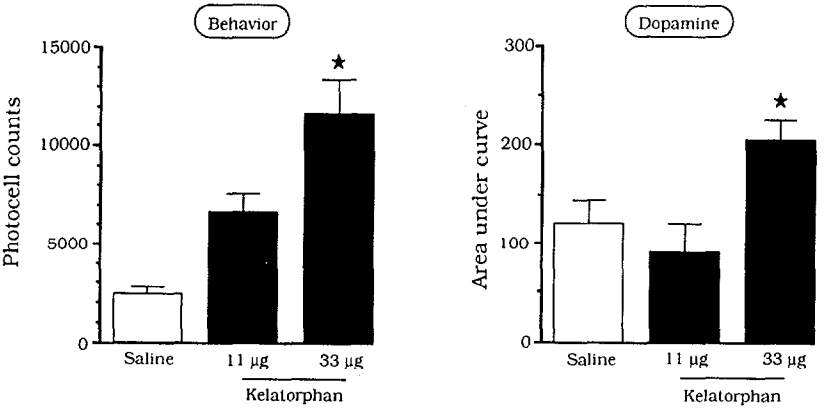


FIGURE 7. *Effects of kelatorphan injected into the VTA on motor activity and extracellular dopamine levels in the nucleus accumbens of rats.*

KEY: ★$p < 0.05$, as compared to control group

cleared from the body despite a clear understanding by an addict of the negative consequences of this decision on his social life. Thus, many patients need rehabilitation or "habilitation," and, unless these aids are included as part of therapy, relapse is almost inevitable.

There are noticeable individual differences in the amount of drug exposure and the environmental conditions required for each user to become dependent on drugs of abuse. Pharmacogenetic studies with drugs of abuse are proliferating, and many genetic animal models are now available for studies of the mechanisms of action of a variety of drugs (Crabbe and Belknap 1992). In a recent study, it has been shown that genotype significantly affects the voluntary consumption of morphine in mice (Belknap et al. 1993). Nevertheless, at this time, there is no scientific evidence that there exists a type of personality that is prone to addiction. It has been proposed that the craving and self-administration of drugs could be explained either by a preexisting deficit in the endogenous opioid system or by a deficit that could occur after chronic administration of opiates. These possibilities provide a good justification for the use of maintenance treatments. Numerous pharmacological agents have begun to be explored on morphine abstinence syndrome in animals, such as *N*-methyl-D-Aspartate (NMDA) antagonists, benzodiazepine agonists, adenosine agonists, α_2 agonists, or endogenous opioids and their analogs, all of which are able to reduce the

short-term acute withdrawal syndrome significantly (Bhargava, this volume). Nevertheless, although early abstinence syndrome may be an Important clinical problem and though it may be quite uncomfortable, it usually is not life threatening. In the treatment of addiction, the most difficult aspect is the protracted abstinence syndrome, one of the main factors contributing to relapse. Indeed, the first days after cessation of prolonged drug use leads to acute withdrawal syndrome, which consists of physiological changes (i.e., agitation, hyperalgesia, tachycardia, hypertension, diarrhea, and vomiting) and a variety of phenomena (i.e., cardiovascular, visceral, thermoregulatory, and subjective changes) or depressive state may persist for months or more after the last dose of opiate.

Thus, the main challenge in the management of opioid addiction is to develop a pharmacotherapy to minimize the short-term withdrawal syndrome and protracted opiate abstinence syndrome. Several treatments of opioid dependence are used clinically (review in O'Brien 1993). Maintenance is a treatment that can be used for opiate addicts who persistently return to opiate use after repeated attempts at detoxification and drug-free treatments. The most commonly used opioid for maintenance is the long-acting agonist methadone. Methadone maintenance reduces the euphoria produced by the heroin due to cross-tolerance and results in significant reductions in heroin use and often its complete elimination. Moreover, the legal availability of methadone permits rehabilitation of the patient, who can focus his attention on constructive activities, and psychotherapies. Nevertheless, certain problems are associated with the use of methadone for maintenance. Like heroin, methadone is a drug, and even if the methadone withdrawal syndrome is less acute than that seen with heroin, it is much longer lasting. This constitutes a serious problem for long-term methadone-maintained patients who wish to end the maintenance phase of treatment. Moreover, chronic methadone use can lead to biochemical and physiological alterations of important pathways (review in Kreek and Hartman 1982).

The use of a more "physiological" maintenance treatment by increasing the level of endogenous opioid peptides could be an interesting new approach in the treatment of drug abuse. Thus, the pharmacotherapy of opiate addiction can be one of the potential clinical applications of the enkephalin-degrading enzyme inhibitors. Indeed, the NEP inhibitors phosphoramidon, thiorphan, and acetorphan and the mixed inhibitor phelorphan have been shown to minimize the severity of the naloxone-

precipitated morphine withdrawal syndrome in rats and mice (Dzoljic et al. 1986; Haffmans and Dzoljic 1987; Livingston et al. 1988). In another study, RB 38A was found more effective than kelatorphan and thiorphan (Maldonado et al. 1989). The greater efficiency of the mixed inhibitors probably is due to the resulting greater increase in enkephalins in certain brain regions, especially those enriched in μ opioid receptors, such as periaqueductal gray matter, which also contains high levels of NEP (Waksman et al. 1986) and could be an important site of action for the development of physical morphine dependence (Laschka et al. 1976). Accordingly, local administration of kelatorphan or RB 38A into the periaqueductal gray matter was shown to reduce the severity of the withdrawal syndrome in rats. This result indicates that during the morphine withdrawal syndrome there is a tonic or naloxone-evoked release of opioid peptides, presumably enkephalins, into this structure and that inhibition of their degradation strongly decreases the severity of the withdrawal syndrome (Maldonado et al. 1992).

The results obtained after i.p. administration of the orally active NEP inhibitors acetorphan and SCH 34,826, which decrease the severity of the naloxone-precipitated withdrawal syndrome in morphine-dependent rodents (Dzoljic et al. 1992; Livingston et al. 1988), suggest that these inhibitors are promising tools in studying modulation of opioid dependence phenomena. More interesting results might be obtained after systemic administration of mixed enkephalin-degrading enzyme inhibitors such as RB 101, which are able to inhibit enkephalin degradation completely.

Previous experiments have shown that chronic treatment with a selective CCK_B receptor antagonist could prevent tolerance to analgesic effects of morphine without affecting morphine-induced physical dependence (Dourish et al. 1990; Watkins et al. 1984; Xu et al. 1992). Nevertheless, the recent demonstration that activation of CCK_B receptors could modulate the opioid system negatively (see above) suggests that selective blockade of these receptors may increase the ability of mixed inhibitors to reduce the opioid withdrawal syndrome. This recently has been confirmed using RB 101 in association with the CCK_B antagonist PD 134,308 (Maldonado et al., submitted).

On the other hand, if enkephalin-degrading enzyme inhibitors could decrease the discomfort of the patient during acute withdrawal syndrome, they also could ameliorate the protracted opiate abstinence, which may include depressive symptoms. Indeed, antidepressant-like effects were

observed after systemic administration of the mixed inhibitors (Baamonde et al. 1992), and these effects are potentiated by selective CCK_B antagonist (Smadja et al., submitted). Thus, combinations of CCK_B antagonists and mixed enkephalin-degrading enzyme inhibitors, which strongly increase endogenous opioid levels, may ameliorate the symptoms of both the short-term and the protracted abstinence syndrome.

CONCLUSIONS AND PERSPECTIVES

The main advantage of modifying the concentration of endogenous peptides by use of peptidase inhibitors is that pharmacological effects are induced only at receptors tonically or phasically stimulated by the natural effectors. Moreover, in contrast to exogenous agonists or antagonists, chronic administration of mixed enkephalin-degrading enzyme inhibitors does not induce changes in the synthesis of the clearing peptidases and in the synthesis of its target peptide precursors, as well as in the secretion of the active peptides (Delay-Goyet et al. 1989*b*; Roques 1988). The goal of discovering analgesics endowed with a potency similar to that of morphine, but devoid of major side effects, now may have been reached with the mixed NEP/APN inhibitors, although these compounds have yet to be evaluated in clinical trials.

Numerous neuromediators are involved in both the control and the transmission of nociceptive messages. Thus, several lines of research also have been developed to obtain new analgesics able to fill the existing gap between opioid analgesics and antalgics. The tachykinin, substance P (sP), is localized in primary afferent nerve fibers involved in transmission of nociception. It has been shown that sP antagonists induce antinociceptive responses, especially in the inflammatory pain model or after noxious chemical stimuli (Garret et al. 1991; Nagahasi et al. 1992; Sakurada et al. 1993). However, these compounds were not active in the hot plate and the tail flick tests, which commonly are used to screen analgesics, thus restricting their potential clinical use.

CCK compounds, especially the selective CCK_B antagonists, also may be interesting drugs in the management of pain. Indeed, even if they do not induce antinociceptive responses alone, they are able to strongly potentiate the antinociceptive effects of the opioids. The clinical implications of this potentiation are very interesting. Indeed, CCK_B antagonists may be useful in potentiating the antinociceptive properties of

exogenous and of endogenous opioids, consequently further reducing the eventual side effects, which may occur after chronic treatment.

The selective NEP inhibitor thiorphan is on the market as a novel antidiarrheal agent. In addition to its antidiarrhetic property, acetorphan has been shown to induce natriuresis and diuresis in humans. The marketing of acetorphan will give important information concerning the possible extension of the clinical indications for NEP inhibitors, for instance, as new antidepressive agents or in management of addiction in association with CCK_B antagonists.

On the other hand, results obtained in rodents suggest that mixed inhibitors such as RB 101 could be used in the treatment of drug abuse. They could represent more efficient compounds than methadone in the treatment of opioid addiction, both because they seem to be unable to trigger dependence and are less susceptible to toxicological problems than the long-lasting agonist methadone. Other maintenance treatments with NMDA antagonists or dynorphin A(1-13) also have been proposed. Nevertheless, these exogenous compounds may have severe side effects and could lead to active metabolites. Thus, the physiological approach corresponding to the inhibition of endogenous opioid peptide catabolism seems to be the most promising way for continuing research. The mixed inhibitors could be administered alone or in combination with the selective CCK_B antagonists to increase the endogenous opioid peptide levels, thus reducing the discomfort of the short-term withdrawal syndrome. The protracted abstinence syndrome also could be ameliorated owing to the antidepressant-like properties of the mixed inhibitors. Thus, the possibility of relapse, the most important problem in the management of opioid addiction, should be minimized.

The recent discovery of the involvement of renal NEP in ANP degradation does not represent a limitation in the use of selective or mixed inhibitors in opioid dependence treatment. Indeed, NEP inhibitors such as acetorphan or candoxatril have no hypotensive effects even in patients with cardiac failure (Northridge et al. 1990). For this reason and because ANP and angiotensin II behave as physiological antagonists, the authors have proposed extension of the concept of mixed NEP/APN inhibitors to dual inhibition of NEP and ACE for the development of a new generation of antihypertensive agents also capable of protecting the wall of injured arteries to restenose (Roques and Beaumont 1990).

REFERENCES

Abbadie, C.; Honoré, P.; Fournié-Zaluski, M.D.; Roques, B.P.; and Besson, J.M. A comparative study of opioids and non-opioids on c-Fos-like immunoreactivity in rat lumbar spinal cord neurons induced by noxious heat stimulation. *Brain Res.* Manuscript submitted for publication.

Al-Rodhan, N.; Chipkin, R.; and Yaksh, T.L. The antinociceptive effects of SCH 32,615, a neutral endopeptidase (enkephalinase) inhibitor, microinjected into the periaqueductal, ventral medulla and amygdala. *Brain Res* 520:123-130, 1990.

Attal, N.; Desmeules, J.; Kayser, V.; Jazat, F.; and Guilbaud, G. Effects of opioids in a rat model of peripheral mononeuropathy. In: Besson, J.M., and Guilbaud, G., eds. *Lesions of Primary Afferent Fibers as a Tool for the Study of Clinical Pain.* Amsterdam: Elsevier Science Publishers, 1991. pp. 245-258.

Baamonde, A.; Daugé, V.; Gacel, G.; and Roques, B.P. Systemic administration of (Tyr-D-Ser(O-Tert-Butyl)-Gly-Phe-Leu-Thr(O-Tert-Butyl), a highly selective delta opioid agonist, induces mu receptor-mediated analgesia in mice. *J Pharmacol Exp Ther* 257:767-773, 1991.

Baamonde, A.; Daugé, V.; Ruiz-Gayo, M.; Fulga, I.G.; Turcaud, S.; Fournié-Zaluski, M.C.; and Roques, B.P. Antidepressant-type effects of endogenous enkephalins protected by systemic RB 101 are mediated by opioid δ and D, dopamine receptor stimulation. *Eur J Pharmacol* 216:157-166, 1992.

Baber, N.S.; Dourish, C.T.; and Hill, D.R. The role of CCK, caerulein, and CCK antagonists in nociception. *Pain* 39:307-328, 1989.

Bateman, R.C., Jr,; Jackson, D.; Slaughter, C.A.; Unnithan, S.; Chai, Y.G.; Moomaw, C.; and Hersh, L.B. Identification of the active site arginine in rat neutral endopeptidase 24.11 (enkephalinase) as Arg 102 and analysis of a glutamine 102 mutant. *J Biol Chem* 264:6151-6157, 1989.

Beaumont, A.; Le Moual, H.; Boileau, G.; Crine, P.; and Roques, B.P. Evidence that both arginine^{102} and arginine^{747} are involved in substrate binding to neutral endopeptidase-24.11. *J Biol Chem* 266:214-224, 1991.

Belknap, J.K.; Crabbe, J.C.; Riggan, J.; and O'Toole, L.A. Voluntary consumption of morphine in 15 inbred mouse strains. *Psychopharmacology* 112:352-358, 1993.

Benchetrit, T.; Bissery, V.; Momon, J.P.; Devault, A.; Crine, P.; and Roques, B.P. Primary structure homologies between two Zn-metallopeptidases, the neutral endopeptidase 24.11 "enkephalinase" and the thermolysin through clustering. *Biochemistry* 27:592-597, 1988.

Bouboutou, R.; Waksman, G.; Devin, J.; Fournié-Zaluski, M.C.; and Roques, B.P. Bidentate peptides: Highly potent new inhibitors of enkephalin degrading enzymes. *Life Sci* 35:1023-1030, 1984.

Boudinot, E.; Denavit-Saubié, M.; Fournié-Zaluski, M.C.; Morin-Surun, M.P.; and Roques, B.P. Respiratory consequences in cats and rats of inhibition of enkephalin-degrading enzymes by kelatorphan. *J Physiol* 406:169P, 1988.

Bourgoin, S.; Le Bars, D.; Artaud, F.; Clot, A.M.; Bouboutou, R.; Fournié-Zaluski, M.C.; Roques, B.P.; Hamon, M.; and Cesselin, F. Effects of kelatorphan and other peptidase inhibitors on the *in vitro* and *in vivo* release of met-enkephalin-like material from the rat spinal cord. *J Pharmacol Exp Ther* 238:360-366, 1986.

Bozarth, M.A. Neural basis of psychomotor stimulant and opiate reward: evidence suggesting the involvement of a common dopaminergic system. *Behav Brain Res* 22:107-116, 1986.

Chaillet, P.; Coulaud, A.; Zajac, J.M.; Fournié-Zaluski, M.C.; Costentin, J.; and Roques, B.P. The mu rather than the delta subtype of opioid receptors appears to be involved in enkephalin induced analgesia. *Eur J Pharmacol* 101:83-90, 1984.

Chaillet, P.; Marçais-Collado, H.; Costentin, J.; Yi, C.C.; De La Baume, S.; and Schwartz, J.C. Inhibition of enkephalin metabolism by, and antinociception activity of, bestatin, an aminopeptidase inhibitor. *Eur J Pharmacol* 86:329-336, 1983.

Chan, W.W.C. L-Leucinethiol, a potent inhibitor of leucine aminopeptidase. *Biochem Biophys Res Commun* 116:297-302, 1983.

Chang, K.J.; Cuatrecasas, P.; Wei, E.T.; and Chang, J.K. Analgesic activity of intracerbroventricular administration of morphiceptin and betacasomorphins: correlation with morphine (mu) receptor binding affinity. *Life Sci* 30:1547-1551, 1982.

Chipkin, R.E.; Berger, J.G.; Billard, W.; Iorio, L.C.; Chapman, R.; and Barnett, A. Pharmacology of SCH34826, an orally active enkephalinase inhibitor analgesic. *J Pharmacol Exp Ther* 245:829-838, 1988.

Chipkin, R.E., and Coffin, V.L. Analgesic and acute central nervous system side effects of the intravenously administered enkephalinase inhibitor SCH 32,615. *Pharmacol Biochem Behav* 38:21-27, 1991.

Crabbe, J.C., and Belknap, J.K. Genetic approaches to drug dependence *Trends Pharmacol Sci* 13:212-219, 1992.

Daugé, V.; Kalivas, P.W.; Duffy, T.; and Roques, B.P. Effects of inhibiting enkephalin catabolism in the VTA on motor activity and extracellular dopamine. *Bruin Res* 599:209-216, 1992.

Daugé, V.; Petit, F.; Rossignol, P.; and Roques, B.P. Use of μ and δ opioid peptides of various selectivity gives further evidence for specific involvement of μ opioid receptors in supraspinal analgesia (tail flick test). *Eur J Pharmacol* 141:171-178, 1987.

Delay-Goyet, P.; Kayser, V.; Zajac, J.M.; Guilbaud, G.; Besson, J.M.; and Roques, B.P. Lack of significant changes in μ, δ opioid binding sites and neutral endopeptidase EC 3.4.24.11 in the brain and spinal cord of arthritic rats. *Neuropharmacology* 28:1341-1348, 1989*a*.

Delay-Goyet, P.; Zajac, J.M.; and Roques, B.P. Effects of repeated treatment with haloperidol on rat striatal neutral endopeptidase EC 3.4.24.11, and on μ and δ opioid binding sites: Comparison with chronic morphine and chronic kelatorphan. *Neurosci Lett* 103:197-202, 1989*b*.

Derrien, M.; Durieux, C.; and Roques, B.P. Antidepressant-like effects of CCK-B antagonists in mice: Antagonism by naltrindole. *Br J Pharmacol* 111:956-960, 1994.

Derrien, M.; Noble, F.; Maldonado, R.; and Roques, B.P. Cholecystokinin-A but not cholecystokinin-B receptor stimulation induces endogenous opioid-dependent antinociceptive effects in the hot plate test in mice. *Neurosci Lett* 160:193-196, 1993.

Devault, A.; Lazure, C.; Nault, C.; Le Moual, H.; Seidah, N.G.; Chretien, M.; Kahn, P.; Powell, J.; Mallet, J.; Beaumont, A.; Roques, B.P.; Crine, P.; and Boileau, C. Amino acid sequence of rabbit kidney neutral endopeptidase 24.11 (enkephalinase) deduced from a complementary DNA. *EMBO J* 6:1317-1322, 1987.

Dickenson, A.H. Recent advances in the physiology and pharmacology of pain: Plasticity and its implications for clinical analgesia. *J Psychopharmacol* 5:342-351, 1991.

Dickenson, A.H.; Sullivan, A.; Feeney, C.; Fournié-Zaluski, M.C.; and Roques, B.P. Evidence that endogenous enkephalins produce δ opioid receptor mediated neuronal inhibitions in rat dorsal horn. *Neurosci Lett* 72:179-182, 1986.

Dickenson, A.H.; Sullivan, A.; and Roques, B.P. Evidence that endogenous enkaphalins and a δ opioid receptor agonist have a common site of action in spinal antinociception. *Eur J Pharmacol* 148:437-439, 1988.

Dourish, C.R.; O'Neill, M.F.; Coughlan, J.; Kitchener, S.J.; Hawley, D.; and Iversen, S.D. The selective CCK-B receptor antagonist L-365,260 enhances morphine analgesia and prevents morphine tolerance in the rat. *Eur J Pharmacol* 176:35-44, 1990.

Dourmap, N.; Michael-Titus, A.; and Costentin, J. Acetorphan, an enkephalinase inhibitor, modulates dopaminergic transmission in rat olfactory tubercle, but not in the nucleus accumbens and striatum. *Eur J Neurosci* 2:783-787, 1990.

Drolet, G.; Van Bockstaele, E.J.; and Aston-Jones, G. Robust enkephalin innervation of the locus coeruleus from the rostral medulla. *J Neurosci* 12:3162-3174, 1992.

Duman, R.S.; Tallman, J.F.; and Nestler, E.J. Acute and chronic opiate-regulation of adenylate cyclase in brain: specific effects in locus coeruleus. *J Pharmacol Exp Ther* 246:1033-1039, 1988.

Dzoljic, M.R.; Bokszanska, A.; Korenhof, A.M.; Kaplan, C.D.; Dzoljic, M.; Rupreht, J.; Zijlstra, F.J.; Brinkman, E.C.A.; and Cappendijk, S.L.T. The effects of orally active enkephalinase inhibitors on morphine withdrawal syndrome. *Neuroreport* 3:637-640, 1992.

Dzoljic, M.R.; Rademaker, B.; Peel-Heisterkamp, A.L.; Ukponmwan, O.E.; and Haffmans, J. Enkephalinase inhibition suppresses naloxone-induced jumping in morphine dependent mice. *Arch Int Pharmacodyn Ther* 283:222-228, 1986.

Elliot, R.L.; Marks, N.; Berg, M.J.; and Portoghese, P.S. Synthesis and biological evaluation of phosphonomidate peptide inhibitors of enkephalinase and angiotensin converting enzyme. *J Med Chem* 28:1208-1215, 1985.

Fang, F.G.; Fields, H.L.; and Lee, N.M. Action at the mu receptor is sufficient to explain the supraspinal analgesic effects of opiates. *J Pharmacol Exp Ther* 238:1039-1044, 1986.

Faris, P.L.; Komisaruk, B.; Watkins, L.R.; and Mayer, D.J. Evidence for the neuropeptide cholecystokinin as an antagonist of opiate analgesia. *Science* 219:310-312, 1983.

Fournié-Zaluski, M.C.; Chaillet, P.; Bouboutou, R.; Coulaud, A.; Chérot, P.; Waksman, G.; Costentin, J.; and Roques, B.P. Analgesic effects of kelatorphan, a new highly potent inhibitor of multiple enkephalin degrading enzymes. *Eur J Pharmacol* 102:525-528, 1984*a*.

Fournié-Zaluski, M.C.; Chaillet, P.; Soroca-Lucas, E.; Costentin, J.; and Roques, B.P. New carboxyalkyl inhibitors of brain "enkephalinase": synthesis, biological activity and analgesic properties. *J Med Chem* 26:60-65, 1983.

Fournié-Zaluski, M.C.; Coric, P.; Turcaud, S.; Lucas, E.; Noble, F.; Maldonado, R.; and Roques, B.P. Mixed-inhibitor-prodrug as a new approach towards systemically active inhibitors of enkephalin degrading enzymes. *J Med Chem* 35:2473-2481, 1992*b*.

Fournié-Zaluski, M.C.; Coulaud, A.; Bouboutou, R.; Chaillet, P.; Devin, J.; Waksman, G.; Costentin, J.; and Roques, B.P. New bidentates as full inhibitors of enkephalin degrading enzymes: Synthesis and analgesic properties. *J Med Chem* 28:1158-1169, 1985.

Fournié-Zaluski, M.C.; Llorens, C.; Gacel, G.; Malfroy, B.; Swerts, J.P.; Lecomte, J.M.; Schwartz, J.C.; and Roques, B.P. Synthesis and biological properties of highly potent enkephalinase inhibitors. In: Brunfeld, K., ed. *Peptides 1980.* Copenhagen: Scriptor, 1981. pp. 476-481.

Fournié-Zaluski, M.C.; Lucas, E.; Waksman, G.; and Roques, B.P. Differences in the structural requirements for selective interaction with neutral metalloendopeptidase (enkephalinase) or angiotensin converting enzyme: Molecular investigation by use of new thiol inhibitors. *Eur J Biochem* 139:267-274, 1984*b*.

Fournié-Zaluski, M.C.; Perdrisot, R.; Gacel, G.; Swerts, J.P.; Roques, B.P.; and Schwartz, J.C. Inhibitory potency of various peptides on enkephalinase activity from mouse striatum. *Biochem Biophys Res Commun* 91:130-135, 1979.

Fournié-Zaluski, M.C.; Soleilhac, J.M.; Turcaud, S.; Lai-Kuen, R.; Crine, P.; Beaumont, A.; and Roques, B.P. Development of [^{125}I]RB104, a new potent inhibitor of neutral endopeptidase-24.11 and its use in detecting nanogram quantities of the enzymes by "Inhibitor Gel Electrophoresis." *Proc Natl Acad Sci U S A* 89:6388-6394, 1992*a*.

Gall, G.; Luterbom, J.; Burks, D.; and Seroogy, K. Co-localization of enkephalin and cholecystokinin in discrete areas of rat brain. *Bruin Res* 403:403-408, 1987.

Garret, C.; Carruette, A.; Fardin, V.; Moussaoui, S.; Peyronel, J.F.; Blanchard, J.C.; and Laduron, P.M. Pharmacological properties of a potent and selective nonpeptide substance P antagonist. *Proc Natl Acad Sci U S A* 88:10208-10212, 199l.

Giros, B.; Gros, C.; Solhonne, B.; and Schwartz, J.C: Characterization of aminopeptidases responsible for inactivating endogenous (Met^5) enkephalin in brain slices using peptidase inhibitors and anti-aminopeptidase M antibodies. *Mel Pharmacol* 29:281-287, 1986.

Gomez-Monterrey, I.; Turcaud, S.; Lucas, E.; Bruetschy, L.; Roques, B.P.; and Fournié-Zaluski, M.C. Exploration of neutral endopeptidase (EC 3.4.24.11) active site by a series of new thiol containing inhibitors. *J Med Chem* 36:87-96, 1993.

Gros, C.; Giros, B.; Schwartz, J.C.; Vlaiculescu, A.; Costentin, J.; and Lecomte, J.M. Potent inhibition of cerebral aminopeptidases by carbaphethiol, a parenterally active compound. *Neuropeptides* 12:111-118, 1988.

Haffmans, J., and Dzoljic, M.R. Inhibition of enkephalinase activity attenuates naloxone precipitated withdrawal symptoms. *Gen Pharmacol* 18:103-105, 1987.

Hambrook, J.M.; Morgan, B.A.; Rance, M.J.; and Smith, C.F.C. Mode of deactivation of the enkephalins by rat and human plasma and rat brain homogenates. *Nature* 263:782-783, 1976.

Hangauer, D.G.; Monzingo, A.F.; and Matthews, B.W. An interactive computer graphics study of thermolysin-catalysed peptide cleavage and inhibition by N-carboxymethyl dipeptides. *Biochemistry* 23:5730-5741, 1984.

Heidbreder, C.; Gewiss, M.; Lallemand, S.; Roques, B.P.; and De Witte, P. Inhibition of enkephalin metabolism and activation of mu- or delta-opioid receptors elicit opposite effects on reward and motility in the ventral mesencephalon. *Neuropharmacology* 31:293-298, 1992.

Hemando, F.; Fuentes, J.A.; Roques, B.P.; and Ruiz-Gayo, M. The CCK-B antagonist L-365,260 elicits antidepressant-type effects in the forced-swim test in mice. *Eur J Pharmacol.* Manuscript submitted for publication.

Hill, R.G.; Hughes, J.; and Pittaway, K.M. Antinociceptive action of cholecystokinin octapeptide (CCK_8) and related peptides in rats and mice: Effects of naloxone and peptidase inhibitors. *Neuropharmacology* 26:289-300, 1987.

Hong, J.S.; Yang, H.Y.T.; Fratta, W.; and Costa, E. Chronic treatment with haloperidol accelerates biosynthesis of enkephalin in rat striatum. *Bruin Res* 160:192-195, 1979.

Hughes, J.; Smith, T.W.; Kosterlitz, H.W.; Fotbergill, L.A.; Morgan, B.A.; and Morris, H.R. Identification of two related pentapeptides from the brain with potent opiate agonist activity. *Nature* 258:577-579, 1975.

Itoh, S.; Katsuura, G.; and Maeda, Y. Caerulein and cholecystokinin suppress ß-endorphin-induced analgesia in the rat. *Eur J Pharmacol* 80:421-425, 1982.

Kayser, V.; Fournié-Zaluski, M.C.; Guilbaud, G.; and Roques, B.P. Potent antinociceptive effects of kelatorphan (a highly efficient inhibitor of multiple enkephalin degrading enzymes) systemically administered in normal and arthritic rats. *Brain Res* 497:94-101, 1989.

Kerr, M.A., and Kenny, A.J. The molecular weight and properties of a neutral metallo-endopeptidase from rabbit kidney brush border. *Biochem J* 137:489-495, 1974.

Kreek, M.J., and Hartman, N. Chronic use of opioids and antipsychotic drugs: Side effects, effects on endogenous opioids, and toxicity. *Ann N Y Acad Sci* 398:151-172, 1982.

Laschka, E.; Teschemacher, H.; Mehrain, P.; and Herz, A. Sites of action of morphine involved in the development of physical dependence in rats. *Psychopharmacologia* 46:141-147, 1976.

Lecomte, J.M.; Costentin, J.; Vlaiculescu, A.; Chaillet, P.; Marçais-Collado, H.; Llorens-Cortes, C.; Leboyer, M.; and Schwartz, J.C. Pharmacological properties of acetorphan, a parenterally active enkephalinase inhibitor. *J Pharmacol Exp Ther* 237:937-944, 1986.

Le Moine, C.; Normand, E.; and Bloch, B. Phenotypical characterization of the rat striatal neurons expressing the D1 dopamine receptor gene. *Proc Natl Acad Sci U S A* 88:4205-4209, 1991.

Le Moual, H.; Devault, A.; Roques, B.P.; Crine, P.; and Boileau, G. Identification of Glutamic Acid 646 as a zinc-coordinating residue in endopeptidase-24.11 *J Biol Chem* 266:15670-15674, 1991.

Livingston, S.J.; Sewell, R.D.E.; Rooney, K.F.; and Smith, H.J. Amelioration of naloxone-precipitated opioid withdrawal symptoms by peripheral administration of the enkephalinase inhibitor acetorphan. *Psychopharmacology* 94:540-544, 1988.

Llorens, C.; Gacel, G.; Swerts, J.P.; Perdrisot, R.; Fournié-Zaluski, M.C.; Schwartz, J.C.; and Roques, B.P. Rational design of enkephalinase inhibitors: substrate specificity of enkephalinase studied from inhibitory potency of various peptides. *Biochem Biophys Res Commun* 96:1710-1715, 1980.

Magnuson, D.S.K.; Sullivan, A.F.; Simonnet, G.; Roques, B.P.; and Dickenson, A.H. Differential interactions of cholecystokinin and FLFQPQRF-NH, with μ and δ opioid antinociception in the rat spinal cord. *Neuropeptides* 16:213-218, 1990.

Maldonado, R.; Daugé, V.; Callebert, J.; Villette, J.M.; Fournié-Zaluski, M.C.; Féger, J.; and Roques, B.P. Comparison of selective and complete inhibitors of enkephalin degrading enzymes on morphine withdrawal syndrome. *Eur J Pharmacol* 165:199-207, 1989.

Maldonado, R.; Daugé, V.; Féger, J.; and Roques, B.P. Chronic D_2, but not D_1-dopamine receptors blockade facilitates behavioural responses to endogenous enkephalins protected by kelatorphan administered intraaccumbens in rats. *Neuropharmacology* 29:214-2323, 1990*a*.

Maldonado, R.; Derrien, M.; Noble, F.; and Roques, B.P. Association of the peptidase inhibitor RB 101 and a CCK-B antagonist strongly enhances antinociceptive responses. *Neuroreport* 4:947-950, 1993.

Maldonado, R.; Féger, J.; Fournié-Zaluski, M.C.; and Roques, B.P. Differences in physical dependence induced by selective μ or δ opioid agonists and by endogenous enkephalins protected by peptidase inhibitors. *Brain Res* 520:247-254, 1990*b*.

Maldonado, R.; Fournié-Zaluski, M.C.; and Roques, B.P. Attenuation of the morphine withdrawal syndrome by inhibition of catabolism of endogenous enkephalins in the periaqueductale gray matter. *Naunyn Schmiederbergs Arch Pharmacol* 345:466-472, 1992.

Maldonado, R.; Valverde, O.; Ducos, B.; Coric, P.; and Roques, B.P. Inhibition of morphine withdrawal syndrome by the association of a peptidase inhibitor and a CCK-B antagonist. *Br J Pharmacol.* Manuscript submitted for publication.

Maldonado, R.; Valverde, O.; Turcaud, S.; Fournié-Zaluski, M.C.; and Roques, B.P. Antinociceptive response induced by mixed inhibitors of enkephalin catabolism in peripheral inflammation. *Pain,* in press.

Malfroy, B.; Swerts, J.P.; Guyon, A.; Roques, B.P.; and Schwartz, J.C. High-affinity enkephalin-degrading peptidase in mouse brain and its enhanced activity following morphine. *Nature* 276:523-526, 1978.

Matsuoka, I.; Maldonado, R.; Defer, N.; Noel, F.; Hanoune, J.; and Roques, B.P. Region specific increase in brain type VIII adenylyl cyclase mRNA associated with behavioral expression of morphine withdrawal in mice. *Eur J Pharmacol,* in press.

Matthews, B.W. Structural basis of the action of thermolysin and related zinc peptidases. *Acc Chem Res* 21:333-340, 1988.

McLennan, A., and Maier, S.F. Coping and the stress-induced potentiation of stimulant stereotypy in the rat. *Science* 219:1091-1093, 1983.

Meucci, E.; Delay-Goyet, P.; Roques, B.P.; and Zajac, M.J. Binding *in vivo* of selective μ and δ opioid agonists: Opioid receptor occupancy by endogenous enkephalins. *Eur J Pharmacol* 171:167-178, 1989.

Meynadier, J.; Dalmas, S.; Lecomte, J.M.; Gros, C.; and Schwartz, J.C. Potent analgesic effects of inhibitors of enkephalin metabolism administered intrathecally to cancer patients. *Pain* 2:201-206, 1988.

Michael-Titus, A.; Dourmap, N.; Caline, H.; Costentin, J.; and Schwartz, J.C. Role of endogenous enkephalins in locomotion and nociception studied with peptidase inhibitors in two inbred strains of mice. *Neuropharmacology* 28:117-122, 1989.

Moran, T.H.; Robinson, P.H.; Goldrich, M.S.; and McHugh, P.R. Two brain cholecystokinin receptors: Implication for behavioral actions. *Brain Res* 362:175-179, 1986.

Morin-Surun, M.P.; Boudinot, E.; Fournie-Zaluski, M.C.; Champagnat, J.; Roques, B.P.; and Denavit-Saubié, M. Control of breathing by endogenous opioid peptides: Possible involvement in sudden infant death syndrome. *Neurochem Int* 20:103-107, 1992.

Morris, B.J., and Herz, A. Control of opiate receptor number in vivo: Simultaneous K-receptor down-regulation and μ-receptor up-regulation following chronic agonist/antagonist treatment. *Neuroscience* 29:433-442, 1989.

Morris, B.J.; Höllt, V.; and Herz, A. Opioid gene expression in rat striatum is modulated via opioid receptors: Evidence from localized receptor inactivation. *Neurosci Lett* 89:80-84, 1988.

Morton, C.R.; Zhao, Z.Q.; and Duggan, A.W. Kelatorphan potentiates the effect of Met^5-enkephalin in the substantia gelatinosa of the cat spinal cord. *Eur J Pharmacol* 140:195-201, 1987.

Nagahasi, A.; Asai, R.; Kanai, Y.; Murase, A.; Tsuchiya-Nakagaki, M.; Nakagaki, T.; Shieh, T.C.; and Taniguchi, K. Non-specific activity of (±)-CP-96,345 in models of pain and inflammation. *Br J Pharmacol* 107:273-275, 1992.

Nestler, E.J. Molecular mechanisms of drug addiction. *J Neurosci* 12:2439-2450, 1992.

Nestler, E.J., and Tallman, J.F. Chronic morphine treatment increases cyclic AMP-dependent protein kinase activity in the rat locus coeruleus. *Mol Pharmacol* 33:127-132, 1988.

Noble, F.; Blommaert, A.; Fournie-Zaluski, M.C.; and Roques, B.P. A selective CCK-B antagonist potentiates antinociceptive responses induced by mu but not delta opioid receptor activation in the formalin test in mice. *Eur J Pharmacol.* Manuscript submitted for publication.

Noble, F.; Coric, P.; Fournié-Zaluski, M.C.; and Roques, B.P. Lack of physical dependence in mice after repeated systemic administration of the mixed inhibitor prodrug of enkephalin-degrading enzymes, RB 101. *Eur J Pharmacol* 223:91-96, 1992*b*.

Noble, F.; Derrien, M.; and Roques, B.P. Modulation of opioid analgesia by CCK at the supraspinal level. Evidence of regulatory mechanisms between CCK and enkephalin systems in the control of pain. *Br J Pharmacol* 109:1064-1070, 1993*a*.

Noble, F.; Fournié-Zaluski, M.C.; and Roques, B.P. Unlike morphine, the endogenous enkephahns protected by RB 101 are unable to establish a conditioned place preference in mice. *Eur J Pharmacol* 230:139-149, 1993*b*.

Noble, F., and Roques, B.P. Assessment of endogenous enkephalins efficacy in the hot plate test in mice: Comparative study with morphine. *Neurosci Lett.* Manuscript submitted for publication.

Noble, F.; Soleilhac, J.M.; Soroca-Lucas, E.; Turcaud, S.; Fournié-Zaluski, M.C.; and Roques, B.P. Inhibition of the enkephalin-metabolizing enzymes by the first systemically active mixed inhibitor prodrug RB 101 induces potent analgesic responses in mice and rats. *J Pharmacol Exp Ther* 261:181-190, 1992*a*.

Noble, F.; Turcaud, S.; Fournié-Zaluski, M.C.; and Roques, B.P. Repeated systemic administration of the mixed inhibitor of enkephalin-degrading enzymes, RB 101, does not induce either antinociceptive tolerance or cross tolerance with morphine. *Eur J Pharmacol* 223:83-89, 1992*c*.

Northridge, D.B.; Alabaster, C.T.; Connell, J.M.C.; Dilly, S.G.; Lever, A.F.; Jar-dine, A.G.; Barclay, P.L.; Dargie, H.J.; Findlay, I.N.; and Samules, G.M.R. Effects of UK 69578: a novel atriopeptidase inhibitor. *Lancet* 2:591-593, 1989.

Northridge, D.B.; Jardine, A.G.; Findlay, I.N.; Archibald, M.; Dilly, S.; and Dargie, H.J. Inhibition of the metabolism of atrial natriuretic factor causes diuresis and natriuresis in chronic heart failure. *Am J Hypertens* 3:682-687, 1990.

O'Brien, C.P. Opioid addiction. In: Herz, A., ed. *Opioids* Vol. II. Berlin: Springer-Verlag, 1993. pp. 803-823.

Oshita, S.; Yaksh, T.L.; and Chipkin, R. The antinociceptive effects of intrathecally administered SCH 32615, an enkephalinase inhibitor in the rat. *Brain Res* 515:143-148, 1990.

Parsons, C.G., and Herz, A. Peripheral opioid receptors mediating antinociception in inflammation: Evidence for activation by enkephalin-like opioid peptides after cold water swim stress. *J Pharmacol Exp Ther* 255:795-802, 1990.

Perrot, S.; Kayser, V.; Fournié-Zaluski, M.C.; Roques, B.P.; and Guilbaud, G. Antinociceptive effects of systemic PC 12, a prodrug mixed inhibitor of enkephalin-degrading enzymes, in normal and arthritic rats. *Eur J Pharmacol* 241:129-133, 1993.

Phillips, A.G., and Le Piane, F.G. Reward produced by microinjection of (D-Ala^2), Met^5-enkephalinamide into the ventral tegmental area. *Behav Brain Res* 5:255-229, 1982.

Pohl, M.; Benoliel, J.J.; Bourgoin, S.; Lombard, M.C.; Mauborgne, A.; Taquet, H.; Carayon, A.; Besson, J.M.; Cesselin, F.; and Hamon, M. Regional distribution of calcitonin gene related peptide-, substance P-, cholecystokinin-, Met^5-enkephalin- and dynorphin A (1-8)-like materials in the spinal cord and dorsal root ganglia of adult rats: Effects of dorsal rhizotomy and neonatal capsaicin. *J Neurochem* 55:1122-1130, 1990.

Porreca, F.; Lopresti, D.; and Ward, S.J. Opioid agonist affinity in the guinea-pig ileum and mouse vas deferens. *Eur J Pharmacol* 179:129-139, 1990.

Roques, B.P. Physiological role of endogenous peptide effectors studies with peptidase inhibitors. *Kidney Int* 34:S27-S33, 1988.

Roques, B.P. Zinc metallopeptidases: Active site structure and design of selective and mixed inhibitors: New approaches in the search for analgesics and anti-hypertensives. *Biochem Soc Trans* 21:678-685, 1993.

Roques, B.P., and Beaumont, A. Neutral endopeptidase-24.11 inhibitors: From analgesics to antihypertensives. *Trends Pharmacol Sci* 11:245-249,1990.

Roques, B.P.; Daugé, V.; Gacel, G.; and Fournié-Zaluski, M.C. Selective agonists and antagonists of delta opioid receptors and inhibitors of enkephalin metabolism: Potential use in treatment of mental illness. In: Shagass, C.; Josiassen, R.C.; Bridger, H.W.; Weiss, K.J.; Stoff, D.; and Simpon, G.M., eds. *Biological Psychiatry, Developments in Psychiatry.* Vol. 7. New York: Elsevier, 1985. pp. 287-289.

Roques, B.P., and Fournié-Zaluski, M.C. Enkephalin degrading enzyme inhibitors: A physiological way to new analgesics and psychoactive agents. In: Rapaka, R.S., and Hawks, R.L., eds. *Opioid Peptides: Molecular, Pharmacology, Biosynthesis, and Analysis.* National Institute on Drug Abuse Research Monograph 70. DHHS Pub. No. (ADM)87-1455. Washington, DC: Supt. of Docs., U.S. Govt. Print. Off., 1987. pp. 128-154.

Roques, B.P.; Fournié-Zaluski, M.C.; Soroca, E.; Lecomte, J.M.; Malfroy, B.; Llorens, C.; and Schwartz, J.C. The enkephalinase inhibitor thiorphan shows antinociceptive activity in mice. *Nature* 288:286-288, 1980.

Roques, B.P.; Lucas-Soroca, E.; Chaillet, P.; Costentin, J.; and Fournié-Zaluski, M.C. Complete differentiation between "enkephalinase" and angiotensin converting enzyme inhibition by retro-thiorphan. *Proc Natl Acad Sci U S A* 80:3178-3182, 1983.

Roques, B.P.; Noble, F.; Daugé, V.; Fournié-Zaluski, M.C.; and Beaumont, A. Neutral endopeptidase 24.11: Structure, inhibition, and experimental and clinical pharmacology. *Pharmacol Rev* 45:87-146, 1993.

Ruiz-Gayo, M.; Baamonde, A.; Turcaud, S.; Fournié-Zaluski, M.C.; and Roques, B.P. In vivo occupation of mouse brain opioid receptors by endogenous enkephalins: Blockade of enkephalin degrading enzymes by RB 101 inhibits [^{3}H]diprenorphine binding. *Brain Res* 571:306-312, 1992.

Rupreht, J.; Ukponmwan, O.E.; Admiraal, P.V.; and Dzoljic, M.R. Effect of phosphoramidon, a selective enkephalinase inhibitor, on nociception and behaviour. *Neurosci Lett* 41:331-335, 1983.

Sakurada, T.; Katsumata, K.; Yogo, H.; Tan-No, K.; Sakurada, S.; and Kisara, K. Antinociception induced by CP 96,345, a non-peptide NK-1 receptor antagonist, in the mouse formalin and capsaicin tests. *Neurosci Lett* 151:142-145, 1993.

Schmidt, C.; Peyroux, J.; Noble, F.; Fournié-Zaluski, M.C.; and Roques, B.P. Analgesic responses elicited by endogenous enkephalins (protected by mixed peptidase inhibitors) in a variety of morphine-sensitive noxious tests. *Eur J Pharmacol* 192:253-262, 1991.

Scott Young, W., III; Bonner, T.I.; and Brann, M.R. Mesencephalic dopamine neurons regulate the expression of neuropeptide mRNAs in the rat forebrain. *Proc Natl Acad Sci U S A* 83:9827, 1986.

Seymour, A.A.; Fennell, S.A.; and Swerdel, J.N. Potentiation of renal effects of atrial natriuretic factor-(99-126) by SQ 29072. *Hypertension* 14:87-97, 1989.

Shook, J.E.; Pelton, J.T.; Lemck, P.K.; Porreca, F.; Hruby, V.; and Burks, T.F. Opioid antagonist properties of a cyclic somatostatin octapeptide in vivo: Identification of mu receptor-related functions. *J Pharmacol Exp Ther* 242:1-7, 1987.

Smadja, C.; Maldonado, R.; Turcaud, S.; Fournié-Zaluski, M.C.; and Roques, B.P. Modulation of antidepressant-like effects induced by endogenous enkephalins through CCK-B receptors. *Psychopharmacology*. Manuscript submitted for publication.

Stein, C.; Gramsch, C.; and Herz, A. Intrinsic mechanisms of antinociception in inflammation: Local opioid receptors and ß-endorphin. *J Neurosci* 10:1292-1298, 1990.

Stein, C.; Hassan, A.H.S.; Lehrberger, K.; Giefing, J.; and Yassouridis, A. Local analgesic effect of endogenous opioid peptides. *Lancet* 342:321-324, 1993.

Stein, C.; Millan, M.J.; Shippenberg, T.S.; Peter, K.; and Hem, A. Peripheral opioid receptors mediating antinociception in inflammation: Evidence for involvement of mu, delta and kappa receptors. *J Pharmacol Exp Ther* 248:1269-1275, 1989.

Stein, C.; Millan, M.J.; Yassouridis, A.; and Herz, A. Antinociceptive effects of μ- and κ-agonists in inflammation are enhanced by a peripheral opioid receptor-specific mechanism. *Eur J Pharmacol* 155:255-264, 1988.

Stephenson, S.L., and Kenny, A.J. The hydrolysis of α human atrial natriuretic peptide by pig kidney microvillar membranes is initiated by endopeptidase 24.11. *Biochem J* 243:183-187, 1987.

Stinus, L.; Winnock, M.R.; and Kelley, A.E. Chronic neuroleptics treatment and mesolimbic dopamine denervation induce behavioral supersensitivity to opiates. *Psychopharmacology* 85:323-328, 1985.

Sullivan, A.F.; Dickenson, A.H.; and Roques, B.P. δ opioid mediated inhibitions of acute and prolonged noxious-evoked responses in rat dorsal horn neurones. *Br J Pharmacol* 98:1039-1049, 1989.

Sybertz, E.J.; Chiu, P.J.S.; Vemulapalli, S.; Watkins, R.; and Haslanger, M.F. Atrial natriuretic factor-potentiating and hypertensive activity of SCH 34826, an orally active neutral endopeptidase inhibitor. *Hypertension* 15:152-161, 1990.

Thal, L.J.; Sharpless, N.S.; Hirschom, I.D.; Horowitz, S.G.; and Makman, M.H. Striatal Met-enkephalin concentration increases following nigrostriatal denervation. *Biochem Pharmacol* 32:3297-3301, 1983.

Tölle, T.R. Schadrack, J.; Castro-Lopes, J.M.; Evan, G.; Roques, B.P.; and Zielglgansberger, W. Kelatorphan, naloxone and morphine differentially modulate the induction of immediate early genes in the spinal cord of the rat. Supplement. *Eur J Neurosci* 5:192, 1992.

Turner, A.J. Endothelin-converting enzymes and other families of metallo-endopeptidases. *Biochem Soc Trans* 21:697-702, 1993.

Valverde, O.; Maldonado, R.; Fournié-Zaluski, M.C.; and Roques, B.P. CCK-B antagonists very strongly potentiate antinociception mediated by endogenous enkephalins. *J Pharmacol Exp Ther,* in press.

Vanderhaeghen, J.J.; Lotstra, F.; De Mey, J.; and Gilles, C. Immunohistochemical localization of cholecystokinin- and gastrin-like peptides in the brain and hypophysis of the rat. *Proc Natl Acad Sci U S A* 77:1190-1194, 1980.

Waksman, G.; Bouboutou, R.; Devin, J.; Bourgoin, S.; Cesselin, F.; Hamon, M.; Fournié-Zaluski, M.C.; and Roques, B.P. *In vitro* and *in vivo* effects of kelatorphan on enkephalin metabolism in rodent brain. *Eur J Pharmacol* 117:233-243, 1985.

Waksman, G.; Hamel, E.; Fournié-Zaluski, M.C.; and Roques, B.P. Autoradiographic comparison of the distribution of the neutral endopeptidase "enkephalinase" and of mu and delta opioid receptors in rat brain. *Proc Natl Acad Sci USA* 83:1523-1527, 1986.

Watkins, L.R.; Kinscheck, LB.; and Mayer, D.J. Potentiation of opiate analgesia and apparent reversal of morphine tolerance by proglumide. *Science* 224:395-396, 1984.

Watkins, L.R.; Kinscheck, I.B.; and Mayer, D.J. Potentiation of morphine analgesia by the cholecystokinin antagonist proglumide. Brain *Res* 327:169-180, 1985.

Wiesenfeld-Hallin, Z.; Xu, X.J.; Hughes, J.; Horwell, D.C.; and Hökfelt, T. PD134308, a selective antagonist of cholecystokinin type B receptor, enhances the analgesic effect of morphine and synergistically interacts with intrathecal galanin to depress spinal nociceptive reflexes. *Proc Natl Acad Sci U S A* 87:7105-7109, 1990.

Williams, J.T.; Christie, M.J.; North, R.A.; and Roques, B.P. Potentiation of enkephalin action by peptidase inhibitors in rat locus coeruleus in vitro. *J Phamacol Exp Ther* 243:397-401, 1987.

Xie, J.; Soleilhac, J.M.; Renwart, N.; Peyroux, J.; Roques, B.P.; and Fournié-Zaluski, M.C. Inhibitors of the enkephalin degrading enzymes: Modulation of the activity of hydroxamate containing compounds by modification of the C-terminal residue. *Int J Pept Protein Res* 34:246-255, 1989*a*.

Xie, J.; Soleilhac, J.M.; Schmidt, C.; Peyroux, J.; Roques, B.P.; and Fournié-Zaluski, M.C. New kelatorphan related inhibitors of enkephalin metabolism: Improved antinociceptive properties. *J Med Chem* 32:1497-1503,1989*b*.

Xu, X.J.; Wiesenfeld-Hallin, Z.; Hughes, J.; Horwell, D.C.; and Hökfelt, T. CI988, a selective antagonist of cholecystokinin B receptors, prevents morphine tolerance in the rat. *Br J Pharmacol* 105:591-596, 1992.

Yaksh, T.L., and Harty, G.J. Effects of thiorphan on the antinociceptive actions of intrathecal D-Ala^2-Met^5-enkephalin. *Eur J Pharmacol* 79:293-300, 1982.

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Enhanced *N*-Methy-D-Aspartate (NMDA)-Induced Activity Following Morphine: Sensitivity to Sigma and PCP Ligands

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INTRODUCTION

The development of opioid tolerance and dependence appears to depend on activity along pathways involving *N*-methyl-D-Aspartate (NMDA)-induced activity. In support of this, the depolarizing response of cerebral cortical and striatal cells to iontophoretically applied L-glutamate has long been known to be potentiated in morphine tolerant/dependent rats when naloxone is co-administered iontophoretically with this amino acid (Fry et al. 1982). More recently, competitive and noncompetitive antagonists of NMDA have been found to inhibit opioid tolerance and dependence (Marek et al. 1991; Trujillo and Akil 1991) and to inhibit opioid withdrawal (Tanganelli et al. 1991). If NMDA activity is enhanced, it may be due to either an increased release of excitatory amino acids (EAAs) or to an enhanced response to a given concentration of EAAs.

Neuronal substrates supporting tolerance and dependence have not been fully elucidated. Preliminary studies suggest, however, that MK-801 inhibits the development of morphine tolerance by an action at the spinal cord level (Gutstein et al. 1992). Both opioid and EAA receptors in this area perform crucial roles in the regulation of pain, especially at the level of sensory input to the dorsal horn of the spinal cord (Davies and Lodge 1987; Davies and Watkins 1983). Acutely, morphine inhibits NMDA-induced activity in the mouse spinal cord (Aanonsen and Wilcox 1987), which may account, in part, for its antinociceptive effect. Activity mediated by EAAs in the spinal cord, therefore, is a possible target for both opioid analgesia and tolerance to that analgesic effect. Tolerance to morphine implies a change in the interaction between opioids and nociceptive processing characterized by a decreased antinociceptive effect of morphine. One of the hallmarks of morphine withdrawal is hyperalgesia. While this often is visualized as a decreased efficacy of opiates, chronic morphine may result in tolerance by enhancing the action of EAAs along nociceptive pathways. The mechanism by which

phencyclidine (PCP) ligands and competitive antagonists of NMDA inhibit opioid tolerance may be similar to the mechanism underlying their ability to attenuate the development of hyperalgesia induced by various pain models (Dickenson and Sullivan 1990).

The goal of this work has been to test the hypothesis that NMDA-type EAA-induced activity is increased in the spinal cord after morphine treatment and to determine whether that increase is due to enhanced release of EAAs or an enhanced response along pathways that are activated by a given concentration of these endogenously occurring neurotransmitters interacting with NMDA receptors.

Animals were allowed free access to food and water and used strictly in accordance with the Guidelines of the University of Minnesota Animal Care and Use Committee and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Pub. No, [NIH]78-23, revised 1978).

MICRODIALYSIS STUDIES

In vivo microdialysis was used to monitor the release of glutamate, aspartate, and substance P (sP) from the dorsal horn of the lumbar spinal cord of unrestrained and unanesthetized rats to determine whether there is an increased release during naloxone-precipitated withdrawal of morphine pellet-implanted rats. Male rats (275-300 g) were implanted subcutaneously (s.c.) with a 75-mg morphine pellet while under ether anesthesia and 3 days later anesthetized with halothane and implanted with a transverse microdialysis fiber (200 pm diameter, 50,000 MW cutoff, Amicon Vitafiber II) through the dorsal spinal cord via an intrathecal (i.t.) cannula, as described previously (Skilling et al. 1988, 1992). One day postsurgery, rats were perfused with Ringer's solution at 5-6 µl per minute. Samples were collected at 2-minute and 20-minute intervals for EAAs and sP, respectively, and maintained at 5°C or frozen until analysis of amino acids using high performance liquid chromatography or of sP using radioimmunoassay.

The dorsal horn of the spinal cords of rats implanted with a morphine pellet were dialyzed for at least 2 hours to reach equilibrium and to monitor the control release of glutamate, aspartate, glycine, and asparagine. The concentration of these compounds collected in the

dialysis fluid in the three samples immediately prior to challenge with naloxone did not differ from those immediately following an i.t. injection of 50 ng of naloxone or, 40 minutes later, an intraperitoneal (i.p.) injection of 5 mg/kg of naloxone. In similarly prepared rats, the concentration of sP in the dialysis fluid was monitored. Identical injection of naloxone in morphine pellet-implanted rats also failed to change the concentration of sP released into the extracellular fluid, in spite of obvious signs of withdrawal. All morphine-treated rats exhibited defecation, writhing, and agitation following naloxone. The authors previously have measured increases in the release of EAAs in this area of the spinal cord in response to nociceptive stimulation (Skilling et al. 1988), indicating that physiologically relevant concentrations of these substances can be measured. In addition, sP basal concentrations as well as capsaicin-induced increases in sP could be measured rapidly, indicating that the SP assay was sufficiently sensitive and the cannula correctly placed to detect primary afferent C-fiber activity.

These studies suggest that the expression of withdrawal in rats, typically accompanied by hyperalgesia, does not correlate with an enhanced release of the EAAs, glutamate, aspartate, or sP, all of which are putative nociceptive transmitter substances in the dorsal spinal cord.

NMDA-INDUCED BEHAVIORAL ACTIVITY STUDIES

The absence of an enhanced release of putative nociceptive transmitters prompted examination of the possibility that the effect, rather than the release of EAA transmitters, is enhanced in the spinal cord after morphine treatment. Such a change in the potency or efficacy of EAAs may be linked to either the development of tolerance to morphine or the mechanism underlying dependence. The authors examined the effect of morphine pretreatment on the behavioral response evoked by an i.t. injection of NMDA in male mice. I.t.-injected NMDA induces a stereotypical caudally directed biting and scratching behavioral response in mice (Aanonsen and Wilcox 1987; Sun and Larson 1991), providing a highly sensitive and reproducible model of NMDA-precipitated activity in the spinal cord.

All i.t. injections in mice were made at the L5-L6 intervertebral space using a 30-gauge, 0.5-inch disposable needle on a 50-µl Luer tip microsyringe. A volume of 5 µl was used for all i.t. injections. For injections of NMDA, 200 pmol were used, since this dose produces an

intensity of behavioral responses over a 90-second interval that allows monitoring of either increases or decreases in the behavioral response.

Immediately after i.t. injection of EAAs, animals were placed in a large glass cylinder containing approximately 2 cm of bedding. The total number of caudally directed bites and scratches over a 2-minute interval was recorded. Injection of the same volume of vehicle over this time interval has been shown to elicit no increase in behaviors above noninjected control mice and to have no effect on the normal exploratory behavior of the mice (Sun and Larson 1991).

Morphine-injection schedules were used that elicit a known change in the sensitivity to naloxone-induced opioid withdrawal jumping in mice. Acute dependence is produced within 2 hours of a single injection of 10 mg/kg of morphine, as reflected by a decrease in the ED, of naloxone required to precipitate withdrawal jumping in mice (Sofuoglu et al. 1990; Wiley and Downs 1979). Pretreatment of mice with 100 mg/kg of morphine 24 hours prior to challenge with the 10 mg/kg dose of morphine further decreases the ED, of naloxone, suggesting an additional degree of dependence in response to this 24-hour pretreatment (Sofuoglu et al. 1990).

Using these two injection schedules to induce two different degrees of morphine dependence, the authors studied the behavioral response of mice to the phasic (single injection), as well as the tonic (four repeated injections), effects of NMDA administered i.t. Based on the premise that the development of tolerance and dependence is prevented but not reversed by naloxone, it also was determined whether the action of morphine is prevented by naloxone administered immediately prior to morphine or reversed by naloxone administered with NMDA 2 hours after morphine.

Injection of mice with 200 pmol of NMDA it. resulted in an intense biting and scratching behavioral response (table 1) that lasted approximately 90 seconds, as described previously (Sun and Larson 1991). Injected at 2-minute intervals, the intensity of responses to tonically administered NMDA did not change significantly, as indicated by the similar number of behaviors following the fourth injection of NMDA (table 2).

A dose of 10 mg/kg of morphine 2 hours before testing significantly decreased the behavioral response to the first four i.t. injections of

NMDA (table 1). This is consistent with previous reports that NMDA-induced behavior is very sensitive to inhibition by mu-type opioid activity (Aanonsen and Wilcox 1987). Morphine pretreatment had no effect on the intensity of responses to additional injections of NMDA administered via a cannula at 2-minute intervals, as indicated by the number of behaviors in table 2. This would suggest that more tonically evoked NMDA activity is less sensitive to the inhibitory effect of morphine. While morphine itself had little influence on the intensity of this more tonically evoked NMDA activity, the inclusion of 0.1 μg of naloxone with each injection of NMDA resulted in a gradual but dramatic increase in the number of behaviors produced by each of four repeated injections of NMDA in morphine-pretreated mice. Administration of this dose of naloxone with NMDA in the absence of morphine had no effect on the number on NMDA-induced behaviors, indicating that the morphine rather than the naloxone ultimately was responsible for the increased responsiveness to NMDA after the fourth injection. Naloxone merely unmasked this naloxone-irreversible effect of morphine.

To determine whether the facilitation of NMDA-induced activity by morphine was potentiated further by morphine, an additional 100 mg/kg of morphine was administered to mice 24 hours prior to the 10 mg/kg injection of morphine. While this additional injection of a relatively high dose of morphine elicits a great opioid withdrawal jumping behavior in mice upon challenge with naloxone, there was no change in the number of NMDA-induced behaviors in the presence of naloxone, compared to the responses in mice pretreated 24 hours previously with saline. This indicates that neither sensitization nor tolerance develops to this effect of morphine under the conditions described.

Because MK-801 has been used to inhibit the development of tolerance and dependence, its effect on these changes in NMDA-induced activity following morphine also was examined. Pretreatment with 0.3 mg/kg of MK-801 alone, like morphine, inhibited only the first of four injections of NMDA (table 1). While morphine and MK-801 each inhibited NMDA-induced phasic activity when injected alone, there was no effect of this combination of drugs when injected together, either in the absence or presence of naloxone in the NMDA injection. The facilitatory effect of morphine on tonic NMDA activity, unmasked by naloxone (table 2), was not only prevented by MK-801, but the response to this combination was significantly less than the control. This indicates a highly effective protection by MK-801 against this acute effect of morphine on NMDA-induced activity.

TABLE 1. *Number of NMDA-induced behaviors after morphine: Effects of MK-801 and haloperidol*

Treatment[a]	Pretreatment 2 hours before NMDA		
	Saline	MK-801 (0.3 mg/kg)	Haloperidol (0.2 mg/kg)
NMDA (200 pmol, i.t.)	58.3±4.8	3 1.3±5.4*	54.0±3.8
NMDA+1 μg naloxone	65.0±8.4	75.0±8.0	39.0±3.8
NMDA 2 h after 10 mg/kg morphine	24.2±5.4*	46.3±4.5	64.0±4.9
NMDA+naloxone 2 h after morphine	55.3±11.0	34.3±5.0	52.5±8.5

KEY: [a] NMDA was injected either alone or with 1 μg of naloxone i.t. 2 h after an i.p. injection of vehicle, morphine, MK-801, and/or haloperidol as indicated.

* Represents $p < 0.05$ when compared to control mice using analysis of variance (ANOVA)

NOTE: Means (±SEM) of the data are presented. Each group represents at least six mice. Statistical analysis of the results was performed using ANOVA. When presented as percent of control, results were analyzed statistically prior to transformation of data. *P* values less than 0.05 were used as the cutoff to indicate a significant difference between the test group and control values collected the same day.

In contrast to its ability to inhibit the effect of morphine when injected immediately prior to morphine, when injected just 30 minutes before testing with NMDA plus naloxone, MK-801 failed to inhibit the potentiative effect of morphine on the more tonically evoked NMDA

TABLE 2. *Number of behaviors after a fourth injection of NMDA: Effects of morphine, MK-801, and haloperidol*

Treatment[a]	Pretreatment 2 hours before NMDA		
	Saline	MK-801 (0.3 mg/kg)	Haloperidol (0.2 mg/kg)
NMDA (200 pmol i.t.)	47.7±6.2	41.0±5.0	32.0±7.8
NMDA+1 μg naloxone	46.0±6.6	3 1.0±4.2	26.0±8.9
NMDA 2 h after 10 mg/kg morphine	32.4±6.7	19.0±5.0*	32.0±6.3
NMDA+naloxone 2 h after morphine	111.2±11.3*	12.0±6.0”	34.0±4.0

KEY: [a] NMDA was injected in a series of four injections at 2-min intervals either alone or with 1 μ of naloxone i.t. Data represent the response to the fourth injection of NMDA. I.t. injections were made 2 h after an i.p. injection of vehicle, morphine, and MK-801, or haloperidol, or both, as indicated.

* Represents $p < 0.05$ when compared to control mice using one-way ANOVA followed by Scheffe's *F*-test for comparison of individual means

activity elicited by the fourth injection of this amino acid. Because the concentration of MK-801 available after a 30-minute pretreatment is likely as high or higher than that after a 2-hour pretreatment interval, these data suggest that the time of administration with respect to morphine is important in the ability of MK-801 to attenuate morphine's effect. The NMDA-enhancing effect of morphine, therefore, is likely brought about by a pathway that is highly sensitive to PCP ligands but not continuously mediated by a PCP-sensitive synapse.

Based on the ability of ligands acting at the PCP site to interact with sigma receptors, the sensitivity of morphine-evoked changes in NMDA-evoked activity to haloperidol, a sigma ligand and dopamine antagonist, also was tested (Bacopoulos et al. 1978), as well as to spiperone, a butyrophenone similar to haloperidol. Spiperone has a spectrum of actions similar to haloperidol but very little ability to interact with sigma sites (Quik et al. 1979). I.p. injection of 0.2 mg/kg of haloperidol alone, 2.5 hours prior to testing, had no effect on the response to either phasic (table 1) or tonic (table 2) NMDA-induced activity. When administered 30 minutes prior to morphine, haloperidol prevented the opioid-induced inhibition of activity in response to the first injection of NMDA (table 1). Haloperidol also prevented the potentiative effect of morphine on NMDA administered with naloxone. This protective effect was not mimicked by pretreatment with an equivalent dose of spiperone, which has a low potential for interaction with sigma sites, suggesting that the action of haloperidol probably is via an effect involving sigma receptors rather than its ability to inhibit dopaminergic activity.

Like MK-801, when injected 30 minutes prior to NMDA and naloxone, haloperidol also failed to inhibit the facilitatory effect of morphine pretreatment on tonically evoked NMDA activity. This suggests that activity at sigma receptors are necessary for the generation of this potentiative effect on tonic NMDA-induced activity but not for the maintenance of this effect.

A relatively large dose of naloxone (10 mg/kg, i.p.) administered immediately prior to morphine appears to be necessary to prevent the facilitatory effects of 10 mg/kg of morphine on NMDA-induced activity evoked in the presence of naloxone. It, therefore, is possible that the action of morphine on tonic NMDA-induced activity may be brought about by a receptor other than the mu-type opioid site.

NALOXONE-INDUCED WITHDRAWAL JUMPING

Finally, the authors examined the effect of these two compounds that alter morphine's effect on NMDA-induced activity on the acute dependence and withdrawal reflected by the intensity of jumping responses evoked by naloxone in morphine-pretreated mice. Male mice were injected s.c. with 100 mg/kg of morphine sulfate and 3 hours later s.c. administered 16 mg/kg of naloxone. This model is sufficient to elicit a readily quantifiable number of jumping behaviors in response to naloxone

TABLE 3. *Withdrawal jumping in mice after MK-801 or haloperidol*

Drug Dose[a]	(mg/kg)	Percent of control jumps per 15 min	
		30 min before morphine	30 min before naloxone
MK-801	0.3	25.3±14.4*	Unable to count
	0.03	101.3±33	80.7±15.5
Haloperidol	0.2	64.5±10.8	28.2±8.6*
	0.02	NT	82.2±2.6
MK-801 plus Haloperidol	0.03 0.02	79.7±26	45.4±8.1*
MK-801 plus Haloperidol	0.06 0.04	34.5±18	NT

KEY: [a] MK-801 and haloperidol were administered i.p. either 30 minutes prior to 100 mg/kg of morphine or 30 minutes prior to 16 mg/kg of naloxone.

* Represents $p < 0.05$ when compared to control mice using Student's *t*-test

NT Not tested

challenge, as shown in table 3. Using this model of acute opioid dependence, the authors tested the abilities of MK-801 and haloperidol to alter naloxone-induced withdrawal jumping in mice injected 3 hours previously with 100 mg/kg of morphine. MK-801, haloperidol, or saline was administered either 30 minutes prior to morphine or 30 minutes prior to naloxone. The number of jumping behaviors over a 15-minute interval in response to naloxone were quantified.

Compared to saline-injected control mice, a dose of 0.3 mg/kg of MK-801 injected 30 minutes prior to morphine significantly inhibited the number of withdrawal behaviors in this model (table 3). When injected 30 minutes prior to naloxone, MK-801 produced a profound impairment of motor coordination in a fashion that mimicked an overdose of MK-801. Because of this effect of MK-801 on motor activity, the authors were not able to quantify jumping behaviors elicited by subsequent injections with naloxone. Injection of 0.2 mg/kg of haloperidol, either 30 minutes prior to morphine or 30 minutes prior to naloxone, was able to inhibit withdrawal behaviors significantly without deleterious effects on motor activity. When MK-801 and haloperidol were administered 30 minutes before naloxone at doses as low as 0.03 and 0.02 mg/kg (which produced no protection against withdrawal when each was administered alone), the combination of these drugs significantly inhibited withdrawal-induced jumping with no effect on motor activity. The efficacy of this drug interaction suggests a synergistic action by two different mechanisms and requires further investigation.

These data suggest that either MK-801 or haloperidol is able to inhibit withdrawal when administered with morphine. However, when injected after morphine and just 30 minutes prior to naloxone, only haloperidol inhibits opioid withdrawal jumping without causing gross behavioral disturbances.

DISCUSSION

Present data confirm previous data indicating that morphine is a potent inhibitor of phasically evoked NMDA-induced activity (Aanonsen and Wilcox 1987). In the presence of naloxone, however, the action of morphine is to potentiate the response to tonically administered NMDA. These data are consistent with the hypothesis that the development of opioid tolerance and dependence may result from an enhanced action of EAAs. This is demonstrated by the enhanced response to i.t.-administered NMDA plus naloxone in the spinal cord as soon as 2 hours after injection of only 10 mg/kg of morphine. In contrast, present data did not support the hypothesis that morphine withdrawal is mediated by an enhanced release of EAAs or sP from the dorsal spinal cord. This was demonstrated by the inability of naloxone to enhance the concentration of these compounds in the dialysate from morphine pellet-implanted rats.

While pretreatment with either MK-801 or haloperidol 30 minutes prior to 100 mg/kg of morphine effectively inhibited the intensity of withdrawal jumping elicited by naloxone 3 hours later, only haloperidol or a combination of haloperidol plus MK-801 was able to attenuate withdrawal jumping in mice when injected 30 minutes before naloxone. These data suggest the possible involvement of sigma sites in the expression of withdrawal. The enhancing effect of morphine on tonic NMDA-induced activity also is prevented by either MK-801 or haloperidol administered 30 minutes prior to morphine but is not reversed by these drugs when injected 30 minutes prior to challenge with naloxone. The inability of haloperidol and MK-801, administered prior to naloxone, to reverse the facilitatory action of morphine on NMDA-induced behaviors in a fashion similar to their effects on opioid-induced withdrawal jumping would suggest that the modulation of NMDA-induced activity by a sigma site may be distinct from the mechanism involved in the development of dependence or expression of withdrawal. Whether the potentiation of NMDA-induced activity by morphine is related to the development of opioid tolerance or to the hyperalgesic effects of morphine requires further testing.

It is unclear what mediates the motor disturbances observed in response to MK-801 administered to morphine-treated mice. These effects, resembling MK-801 toxicity, are not observed when MK-801 is administered prior to morphine. It is of great interest to note that the ability of MK-801 to inhibit withdrawal jumpings is greater than its ability to inhibit NMDA-induced activity in the absence of morphine. This, too, would suggest a mechanism that is different from noncompetitive antagonism of NMDA.

Changes in the up-regulation of sigma-type activity have been observed in response to 1,3-di(2-tolyl)guanidine (DTG), a relatively nonselective sigma ligand. Those changes were prevented by either MK-801 or PCP (Larson and Sun, in press), similar to the ability of MK-801 to inhibit the effects of morphine in the present study. PCP ligands appear to be able to prevent but not reverse the up-regulation of sigma activity induced by DTG. One might speculate that MK-801 similarly may serve to prevent an up-regulation of sigma activity in response to morphine. Such an involvement of sigma activity during opioid withdrawal is supported by the ability of the relatively low dose of haloperidol, which has very little ability to interact with PCP sites, to inhibit morphine withdrawal. A dose of 10 mg/kg of morphine also has been found to increase the concentration of SP in primary afferent terminals (Kantner et al. 1985)

within 1 hour of injection. A similar up-regulation of other peptides that may serve as endogenous ligands at the sigma site may account for the up-regulation in sigma activity after morphine treatment. Recent data even suggest that sP itself may enhance sigma activity in a fashion that is MK-801 sensitive but not MK-801 reversible (Larson and Sun, in press). Recent reports of interactions between sigma- and NMDA-induced activity (Monnet et al. 1992; Rao et al. 1991) also support the existence of such an interaction.

CONCLUSIONS

There is no increase in the release of EAAs from the dorsal spinal cord of morphine pellet-implanted rats during naloxone-precipitated withdrawal. However, the ability of NMDA to induce behavioral responses is increased when NMDA is administered i.t. together with naloxone in morphine-pretreated mice. While MK-801 and haloperidol each prevent the effect of morphine in this behavioral paradigm, they are unable to reverse the action of morphine when administered 30 minutes prior to NMDA plus naloxone. Potentiation of NMDA by morphine, therefore, is perhaps not related to the abilities of MK-801 and haloperidol to protect against morphine-induced withdrawal jumping, as withdrawal jumping behavior is inhibited by haloperidol administered 30 minutes before naloxone. The impact of this up-regulated NMDA activity following morphine treatment on the development of tolerance needs to be examined.

These data suggest that the behavioral response elicited by specific excitatory compounds administered i.t. may allow elimination or more serious consideration of specific receptor populations as potential mediators of opioid tolerance and withdrawal. These data also suggest that more consideration should be given to the role of sigma sites in the mediation of opioid activity. The inability of naloxone to interact at these sites does not eliminate them from their potential role in dependence and withdrawal, as most characteristics of withdrawal also are insensitive to naloxone.

REFERENCES

Aanonsen, L.M., and Wilcox, G.L. Nociceptive action of excitatory animo acids in the mouse: Effects of spinally administered opioids, phencyclidine and sigma agonists. *J Pharmacol Exp Ther* 243:9-19, 1987.

Bacopoulos, N.G.; Bustos, G.; Redmond, D.E.; Baulu, J.; and Roth, R.H. Regional sensitivity of primate brain dopaminergic neurons to haloperidol: Alterations following chronic treatment. *Brain Res* 157:396-401, 1978.

Davies, S.N., and Lodge, D. Evidence for the involvement of N-methyl-aspartate receptors in "wind-up" of class 2 neurons in the dorsal horn of the rat. *Brain Res* 424:402-406, 1987.

Davies, S.N., and Watkins, J.C. Role of excitatory amino acid receptors in mono- and polysynaptic excitation in the cat spinal cord. *Exp Brain Res* 49:280-289, 1983.

Dickenson, A.H., and Sullivan, A.F. Differential effects of excitatory amino acid antagonists on dorsal horn nociceptive neurons in the rat. *Brain Res* 506:31-39, 1990.

Fry, J.P.; Herz, A.; and Zieglgansberger, W. A demonstration of naloxone precipitated opiate withdrawal on single neurons in the morphine-tolerance dependent rat brain. *Br J Pharmacol* 6:585-592, 1982.

Gutstein, H.B.; Trujillo, K.A.; and Akil, H. MK-801 inhibits the development of morphine tolerance in the rat at spinal sites. Soc *Neurosci Abstr* 1567, 1992.

Kantner, R.M.; Kirby, M.L.; and Goldstein, B.D. Increase in substance P in the dorsal horn during a chemogenic nociceptive stimulus. *Brain Res* 338:196-199, 1985.

Larson, A.A., and Sun, X. Regulation of sigma activity by the amino-terminus of substance P in the mouse spinal cord: Involvement of phencyclidine (PCP) sites not linked to N-methyl-D-aspartate (NMDA) activity. *Neuropharmacology,* in press.

Marek, P.; Ben-Eliyahu, S.; Vaccarino, A.L.; and Liebeskind, J.C. Delayed application of MK-801 attenuates development of morphine tolerance in rats. *Brain Res* 558:163-165, 1991.

Monnet, F.P.; Debonnel, G.; and de Montigny, C. In vivo electrophysiological evidence for a selective modulation of N-methyl-D-aspartate-induced neuronal activation in rat CA, dorsal hippocampus by sigma ligands. *J Pharmacol Exp Ther* 261:123-130, 1992.

Quik, M.; Emson, P.C.; and Joyce, E. Dissociation between the presynaptic dopamine-sensitive adenylate cyclase and [^{3}H]spiperone binding sites in rat substantia nigra. *Brain Res* 167:335-365, 1979.

Rao, T.S.; Mick, S.J.; Cler, J.A.; Emmet, M.R.; Dilworth, V.M.; Contreras, P.C.; Gray, N.M.; Wood, P.L.; and Iyengar, S. Effects of sigma ligands on mouse cerebellar cyclic guanosine monophosphate (cGMP) levels in vivo: Further evidence for a functional modulation of N-methyl-D-aspartate (NMDA) receptor complex-mediated events by sigma ligands. *Brain Res* 561:43-50, 1991.

Skilling, S.R.; Smullin, D.H.; Beitz, A.J.; and Larson, A.A. Extracellular amino acid concentrations in the dorsal spinal cord of freely moving rats following veratridine and nociceptive stimulation. *J Neurochem* 51:127-132, 1988.

Skilling, S.R.; Sun, X.; Kurtz, H.J.; and Larson, A.A. Selective potentiation of NMDA-induced activity and. release of excitatory amino acids by dynorphin: Possible roles in paralysis and neurotoxicity. *Brain Res* 575:272-278, 1992.

Sofuoglu, M.; Sato, J.; and Takemori, A.E. Maintenance of morphine dependence by naloxone in acutely dependent mice. *J Pharmocol Exp Ther* 254:841-846, 1990.

Sun, X., and Larson, A.A. Behavioral sensitization to kainic acid and quisqualic acid in mice: Comparisons to NMDA and substance P responses. *J Neurosci* 11:3111-3123, 1991.

Tanganelli, S.; Aantonelli, T.; Morari, M.; Bianchi, C.; and Beani, L. Glutamate antagonists prevents morphine withdrawal in mice and guinea pigs. *Neurosci Lett* 122:270-272, 1991.

Trujillo, K.A., and Akil, H. Inhibition of morphine tolerance and dependence by the NMDA receptor antagonist MK-801. *Science* 251:85-87, 1991.

Wiley, J.N., and Downs, D.A. Naloxone-precipitated jumping in mice pretreated with acute injection of opioids. *Life Science* 25:797-802, 1979.

ACKNOWLEDGMENTS

This work was supported by U.S. Public Health Service grants NIDA04090, 00124, and 04190 to Dr. Alice A. Larson, The authors would like to thank Dr. Stephen R. Skilling for his effort involved in the completion of the microdialysis studies described in this chapter.

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Dynorphin A: A Rectifying Peptide

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Opioids have been known for their euphoric and analgesic properties for over 2,000 years, but it was not until this century that they came under intensive scientific investigation. Among the major milestones in the development of current knowledge of these drugs are: (1) the synthesis of analogs of morphine and other naturally occurring opioids beginning as early as the late 19th century; (2) the discovery in the early 1970s of opioid receptors, which bind these drugs and initiate their pharmacological actions in the brain; and (3) the discovery a few years later of the endogenous opioid peptides, the natural ligands for these receptors.

The last discovery is one that continues to inspire and invigorate the field, for it identifies the natural substances and their receptors that these age-old drugs, such as morphine, mimic and act on. It also suggests that, presumably, one of the physiological functions performed by endogenous peptides is the regulation of pain. The endogenous opioid peptides soon were shown to fall into three major families: ß-endorphin and related peptides, the enkephalins, and the dynorphins. These families differ in molecular properties, such as the genes that code for them and the enzymes that process them, as well as in their anatomical distribution in the brain. They also differ to some extent in their pharmacological properties.

Though all the endogenous opioid peptides bind to opioid receptors, they differ in their selectivity for different receptor types. Such receptor types, in fact, were to a large extent defined by studies of these peptides. Thus, the enkephalins bind preferentially to δ opioid receptors; dynorphin has some selectivity for κ opioid receptors; and ß-endorphin binds to both μ and δ opioid receptors. While ß-endorphin, like exogenous opioid alkaloids, induces antinociception, dynorphin and some of the enkephalins do not. Each of these peptides also has its own particular constellation of other pharmacological effects.

BACKGROUND AND UNIQUE CHARACTERISTICS

The pharmacological properties of the dynorphins have been of particular interest, for they are quite unique among all known endogenous and exogenous opioids and suggest that this peptide may have the potential for blocking or reversing the most undesirable effects of conventional opioids, particularly their dependence liability. Dynorphin A was first isolated from pituitary glands by Goldstein and colleagues (1979). Both it and a shorter synthetic peptide consisting of its first 13 amino acids, dyn A(1-13), were shown to be more potent than normorphine, Leu-enkephalin, and ß-endorphin in inhibiting electrically induced contractions in the guinea pig ileum and mouse vas deferens, standard assays for opioid agonists. In the ileum preparation, subsequent studies by Huidobro-Toro and colleagues (1981), later confirmed by Goldstein's group, indicated that both dyn A(1-17) and dyn A(1-13) have the properties of κ agonists. On the basis of these findings, it was concluded that dynorphin is the endogenous κ opioid agonist, just as the enkephalins appear to be endogenous δ opioid agonists.

Further studies revealed that dynorphin possesses some unique properties not found in other κ opioid agonists. In particular, it has no antinociceptive potency in mice when given centrally. This lack of effect might be due to rapid degradation but, interestingly, it was shown that dynorphin exhibited other pharmacological effects, indicating that either it or a metabolite remained intact long enough to produce pharmacological effects (Friedman et al. 1981). Specifically, dynorphin was found to inhibit the antinociceptive response to both morphine and ß-endorphin in naive animals.

While this suggested that it might be an endogenous antagonist, a subsequent study revealed that dynorphin had the opposite effect in morphine-tolerant animals, potentiating the antinociceptive effect of both morphine and ß-endorphin (Tulunay et al. 1981) and thus restoring the responsiveness of these animals to morphine. The ED_{50} of morphine in the presence of dynorphin, thereby, is very similar to that for both naive and tolerant animals. Dynorphin seemed to be acting like a "set point" regulator, maintaining the morphine ED_{50} at a more or less constant value.

It subsequently was shown that dynorphin has a modulating influence on other effects of opioid agonists. The peptide was shown to enhance both the hypothermia and the respiratory depression produced by morphine in

naive animals, while it attenuated these effects in morphine-tolerant animals (Woo et al. 1983). When given by itself, dynorphin induced a slight hyperthermia but had no effect on respiration or antinociception.

PHARMACOLOGY AND BIOCHEMISTRY

In their most recent work, Takemori and colleagues (1992) reported that dynorphin increases the ED, of naloxone to precipitate withdrawal jumping in mice. The amount of opioid antagonist required to precipitate withdrawal, as measured by the naloxone ED_{50}, has been shown previously to be inversely proportional to the degree of dependence in the mouse (Way et al. 1969). The ED, of naloxone required to elicit withdrawal jumping was increased substantially by the intravenous (i.v.) administration of dyn A(1-13), implying that the degree of dependence was attenuated. Moreover, the effect could be demonstrated whether the peptide was given before the naloxone challenge or after the withdrawal process had been initiated with naloxone. This observation is surprising because opiate antagonist-induced withdrawal cannot be suppressed easily with opioid agonists in mice (Cheney et al. 1972; Kamei et al. 1973) or in man (Wikler 1980).

There were two other noteworthy points in this study. First, the morphine pellet (75 mg morphine base) was left intact while the mice received the antagonist, which should have assured a high enough concentration of morphine in the central nervous system (CNS) to maintain the dependent state (Patrick et al. 1975). Thus, dynorphin must have had an unusual degree of potency in order to override the excessive amount of morphine already present in the body. Second, the peptide was given by the i.v. route, suggesting either that the site of action for the peptide may be outside the brain or that the peptide can penetrate the blood-brain barrier. Regarding the effects of the route of delivery, it should be pointed out that, in the study discussed earlier, in which dynorphin was shown to have an effect on respiratory depression, the peptide was given by subcutaneous route. In fact, it has been found that dynorphin is effective in reducing withdrawal signs when given either i.v. or intrathecally, but not intracerebroventricularly (Green and Lee 1988); this suggests that it acts at spinal but not supraspinal sites.

In subsequent studies by Takemori and colleagues (1993), a number of dynorphin fragments were used to determine the minimum amino acid sequence that is required to inhibit the expression of signs of morphine

tolerance. A reduction in C-terminus gradually reduced its potency; this was expected because other opioid peptides, such as ß-endorphin, require the C-terminus for pharmacological activity. Much more unexpected, however, was the observation that the N-terminal tyrosine was not required for activity. The minimum sequence that still retained measurable activity was dyn A(2-8). This was quite surprising because this tyrosine, which is common to all endogenous opioid peptides, had been demonstrated in previous studies to be important to their binding and pharmacological activity. Opioid peptides, including dynorphin, exhibit neither binding to brain membranes in vitro nor antinociceptive activity in vivo in the absence of this tyrosine. The ability of destyrosine (des-tyr) dynorphin to inhibit the expression of opioid tolerance and dependence, however, indicates it must be interacting either directly with opioid receptors at an as-yet unidentified receptor or, more likely, indirectly on the opioid system through a nonopioid mechanism. It would be interesting and relevant to an understanding of dynorphin's modulating actions to determine whether the des-tyr peptide is a physiological product or merely a product of nonspecific proteolytic degradation. Interestingly, recent ex vivo studies using dyn A(1-13) revealed that it is degraded rapidly in human blood (within minutes) and that the major long-lasting metabolites were dyn A(2-13) and dyn A(2-12) (Chou et al. 1994). This suggests that the inability of investigators to demonstrate antinociceptive activity by dynorphin may be due to its rapid degradation,

Dynorphin's unique pharmacology also is evident in its interactions with opioid receptors. Although dynorphin seems to be selective as a κ ligand in the in vitro bioassay systems, the authors found that in the brain it interacts in a complex way with the receptors for μ and δ as well as κ ligands (Garzon et al. 1984). Additionally, dynorphin inhibits the high but not the low affinity binding of each receptor and generally affects only Bmax, although with κ compounds it also affects K_d. The IC_{50} values reveal that dynorphin has little or no selective preference for the κ site, having nearly equal affinities for μ and δ sites. These interactions are consistent with its ability to modulate the antinociceptive actions of morphine and ß-endorphin, which act at these sites.

Interestingly, dynorphin was metabolized almost completely within minutes by brain membranes, as determined by a vas deferens assay. In that study, after dynorphin was allowed to equilibrate with brain tissue and had lost activity in the vas deferens, binding of opioid ligands to brain tissue still was inhibited for an additional 45-60 minutes (Garzon et

al. 1984). Further experiments seemed to rule out the role of dynorphin metabolites in this effect since smaller fragments, such as dyn A(1-8) or dyn A(6-17), did not inhibit opiate ligand binding. It appears, therefore, that dynorphin remains active for a significant length of time in vivo (Jen et al. 1983). This finding of long-lasting inhibition of binding is in agreement with the original report of Goldstein and colleagues (1979). Apparently, dynorphin has a very slow rate of dissociation from opioid receptors.

Other studies also indicate that dynorphin is not simply a κ agonist. One of the unique properties distinguishing κ drugs, such as EKC, from morphine and related opioids is their inability to prevent morphine withdrawal in monkeys (Swain and Seevers 1974) and in man (Jasinski 1977). In contrast, as noted above, dynorphin can suppress morphine withdrawal in both systems. Furthermore, dynorphin did not produce ataxia and muscle relaxation in these addicted monkeys, as has been reported for κ drugs.

CLINICAL RELEVANCE

These effects of dynorphin are not merely scientifically interesting but also are clinically promising. A significant problem with the long-term use of opioids as painkillers—for example, in the terminally ill—is that, as tolerance to their effects increases, larger and larger doses must be used until a point is reached at which full pain relief is not possible. Moreover, since little tolerance develops to respiratory depression or constipation, the use of increasingly higher doses of these drugs becomes unsafe. If dynorphin simultaneously can increase the antinociceptive potency of morphine and reduce its respiratory depressive effects in the tolerant system, then it could be used as an adjunct compound, thereby providing safe pain relief to patients that have been on opioids for long periods of time.

Studies with both monkeys (Aceto et al. 1982) and human heroin addicts (Wen and Ho 1982), in fact, have confirmed this promise by demonstrating that dynorphin suppresses the withdrawal symptoms from morphine abstinence apparently without inducing any major side effects. Another study with rodents, moreover, found that even after dynorphin administration was terminated the withdrawal still was suppressed (Khazan et al. 1983). If this finding can be extended to humans, then in principle dynorphin could be used not simply to substitute for

morphine-as is the basis for methadone therapy, the most widely used treatment for opioid addiction now in effect—but also to promote a genuine cure for the problem, in which no exogenous drug is required. Currently, dynorphin is being tested in humans at several medical institutions in the United States, and the findings do indicate that it is medically safe at doses sufficient to produce its modulating effects (Balla et al. 1993).

CONCLUSION

The unique properties of the dynorphins discovered so far may have tremendous significance for understanding how this endogenous opioid system normally functions. Clearly, the opioid system must be under some form of regulation that prevents it from going out of its normal state of balance. This balance is lost, for example, when an animal consumes an exogenous opioid chronically and becomes dependent on it. The current data available on dynorphin A and its active metabolites suggest that this peptide group plays a role in maintaining the endogenous opioid system in a state of balance; this homeostasis, perhaps, is maintained through variations in the levels and activities of dynorphin in certain critical areas of the CNS.

Dynorphin clearly is unique among the endogenous opioid peptides in the body. It acts as neither a classical agonist nor an antagonist, yet has the ability to modulate in a specific fashion the activity of other opioids. Furthermore, the nature of this modulation depends on the state of the animal. In naive animals, dynorphin inhibits the antinociceptive action of opioids, yet it restores the sensitivity of opioid antinociceptive action when the animals are tolerant to morphine. Thus, dynorphin behaves like a rectifying peptide. Dynorphin also can inhibit the expression of morphine dependence by completely substituting for morphine during morphine withdrawal, yet alone it cannot induce morphine-like dependence (Lee et al., unpublished data). Though dynorphin has some preference for the κ opioid receptor, whether any of these actions of dynorphin are mediated through κ receptors is not clear. In any case, the fact that des-tyr peptides, which do not bind to κ receptors, are as active as tyrosine-containing ones suggests the involvement of some unknown opioid or nonopioid systems.

REFERENCES

Aceto, M.D.; Dewey, W.L.; Chang, J.K.; and Lee, N.M. Dynorphin-l-13 substitutes for morphine in addicted rhesus monkeys. *Eur J Pharmacol* 83:139-142, 1982.

Balla, J.; Goli, V.; Urban, B.; Harris, T.; Gayam, R.; and Krishnan, R. "The Effects of Dynorphin on Pain in Chronic Pain Patients Treated with Methadone." Paper presented at the Seventh World Congress on Pain, Paris, 1993.

Cheney, D.L.; Judson, B.A.; and Goldstein, A. Failure of an opiate to protect mice against naloxone-precipitated withdrawal. *J Pharmacol Exp Ther* 182:189-194, 1972.

Chou, J.Z.; Maisonneuve, I.M.; Chait, B.T.; and Kreek, M. J. Study of dynorphin A(1-17) in vivo processing in rat brain by microdialysis and matrix-assisted laser desorption mass spectrometry. In: Harris, L.S., ed. *Problems of Drug Dependence, 1993: Proceedings of the 55th Annual Scientific Meeting of the Committee on the Problems of Drug Dependence, The College* on *Problems of Drug Dependence, Inc.* Vol. II, *Abstracts.* National Institute on Drug Abuse Research Monograph 141. NIH Pub. No. 94-3749. Washington, DC: Supt. of DOCS., U.S. Govt. Print. Off., 1994. p. 240.

Friedman, H.J.; Jen, M.F.; Chang, J.K.; Lee, N.M.; and Loh, H.H. Dynorphin: A possible modulatory peptide on morphine or beta-endorphin analgesia in mouse. *Eur J Pharmacol* 69:351-360, 1981.

Garzon, J.G.; Sanchez-Blazquez, P.; Gerhart, J.; Loh, H.H.; and Lee, N.M. Dynorphin-l-13: Interaction with other ligand bindings in vitro. *Brain Res* 302:392-396, 1984.

Goldstein, A.; Tachibana, S.; Lowney, L.I.; Hunkapiller, M.; and Hood, L. Dynorphin-(l-13): An extraordinarily potent opioid peptide. *Proc Natl Acad Sci U S A* 76:6666-6670, 1979.

Green, P.G., and Lee, N.M. Dynorphin A-(1-13) attenuates withdrawal in morphine-dependent rats: Effect of route of administration. *Eur J Pharmacol* 145:267-272, 1988.

Huidobro-Toro, J.P.; Yoshimura, K.; Lee, N.M.; Loh, H.H.; and Way, E.L. Dynorphin interaction at the kappa-opiate site. *Eur J Pharmacol* 72:265-266, 1981.

Jasinski, D.R. Assessment of abuse potentiality of morphine-like drugs (methods used in man) In: Martin, W.R., ed. *Drug Addiction.* Berlin: Springer-Verlag, 1977. pp. 218-258.

Jen, M.F.; Garzon, J.; Loh, H.H.; and Lee, N.M. Dynorphin-(1-13): The effect of in vivo treatment on opiate binding in vitro. *Eur J Pharmacol* 91:95-99, 1983.

Kamei, C.; Shimomura, K.; and Ueki, S. Significance of withdrawal jumping response in predicting physical dependence in mice. *Jpn J Pharmacol* 23:421-426, 1973.

Khazan, N.; Young, G.A.; and Calligaro, D. Self-administration of Dynorphin-[1-13] and D-Ala^2-Dynorphin-[1-11] (kappa opioid agonists) in morphine (mu opioid agonist)-dependent rats. Supplement I. *Life Sci* 33:559-562, 1983.

Patrick, G.A.; Dewey, W.L.; Spaulding, T.C.; and Harris, L.S. Relationship of brain morphine levels to analgesic activity in acutely treated mice and rats and in pellet-implanted mice. *J Pharmacol Exp Ther* 193:876-883, 1975.

Swain, H.H., and Seevers, M.H. "Addendum: Evaluation of New Compounds for Morphine-Like Physical Dependence in Rhesus Monkeys." Report of the 36th Annual Scientific Meeting of the Committee on Problems of Drug Dependence, NAS-NRC, 1974. pp. 1168-1195.

Takemori, A.E.; Loh, H.H.; and Lee, N.M. Suppression by dynorphin A-(1-13) of the expression of opiate withdrawal and tolerance in mice. *Eur J Pharmacol* 221:223-226, 1992.

Takemori, A.E.; Loh, H.H.; and Lee, N.M. Suppression by Dynorphin A and [Des-tyr^1]Dynorphin A peptides of the expression of opiate withdrawal and tolerance in morphine-dependent mice. *J Pharmacol Exp Ther* 266:121-124, 1993.

Tulunay, F.C.; Jen, M.F.; Chang, J.K.; Loh, H.H.; and Lee, N.M. Possible regulatory role of dynorphin on morphine- and endorphin-dependent analgesia. *J Pharmacol Exp Ther* 219:296-298, 1981.

Way, E.L.; Loh, H.H.; and Shen, F.H. Simultaneous quantitative assessment of morphine tolerance and physical dependence. *J Pharmacol Exp Ther* 167:1-8, 1969.

Wen, H.L., and Ho, W.K.K. Suppression of withdrawal symptoms by dynorphin in heroin addicts. *Eur J Pharmacol* 82:183-186, 1982.

Wikler, A. *Opioid Dependence: Mechanisms and Treatment.* New York Plenum Press, 1980.

Woo, S.K.; Tulunay, F.C.; Loh, H.H.; and Lee, N.M. Effect of dynorphin 1-13 and related peptides on respiratory rate and morphine-induced respiratory rate depression. *Eur J Pharmacol* 86:117-122, 1983.

ACKNOWLEDGMENTS

This work was supported in part by National Institute on Drug Abuse research grant 02643 and Research Scientist award K05-DA0002.

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Inhibitors of Nitric Oxide Synthase and the Opioid Withdrawal Syndrome

Edythe D. London, Alane S. Kimes, and D. Bruce Vaupel

INTRODUCTION

Nitric oxide (NO) is a free radical gas that is purported to have a neurotransmitter function (Garthwaite 1991; Garthwaite et al. 1988). The production of NO has been linked to activity of the *N*-methyl-D-Aspartate (NMDA)-preferring subtype of glutamate receptor (i.e., NMDA receptor) because activation of these receptors in brain initiates a sequence of events that culminates in the production of NO. Receptor activation facilitates the entry of Ca^{2+} into the cell and, consequently, activates calmodulin. Calmodulin binds to the enzyme nitric oxide synthase (NOS), which uses nicotinamide adenine dinucleotide phosphate as a co-factor in catalyzing the conversion of L-arginine to L-citrulline and NO. Since NO is diffusable across cell membranes, it can function as an intercellular mediator.

Several reports in 1991 suggested that the NMDA receptor has a function in the development and expression of opioid dependence and tolerance. In the first of these studies, dizocilpine, a noncompetitive antagonist of the NMDA receptor, was administered to rats over a 10-day period when morphine also was administered (Trujillo and Akil 1991). Dizocilpine attenuated the development of tolerance to morphine analgesia without altering the acute analgesic effects of morphine, and it antagonized the appearance of escape jumps, a sign of opioid dependence. The antagonism by dizocilpine of the opioid withdrawal syndrome in rats was confirmed in a subsequent study in which a wide range of withdrawal signs was monitored; pretreatment with LY274614, a competitive antagonist of the NMDA receptor, also reduced the behavioral signs of opioid withdrawal (Rasmussen et al. 1991*a*). In a subsequent study, kynurenic acid, a nonselective antagonist of excitatory amino acid (EAA) receptors, was given intracerebroventricularly or subcutaneously (s.c.) to opioid-dependent rats (Rasmussen et al. 1991 *b)*. The antagonist suppressed abstinence signs when it was administered by either route, supporting findings with specific antagonists of the NMDA receptor and

suggesting that EAAs play a role both centrally and peripherally in mediating the opioid abstinence syndrome.

Despite these observations, which suggest that potential treatments for opioid dependence might be directed at sites on the NMDA receptor complex, clinical applications of direct interactions with the NMDA receptor are problematic. In this regard, competitive antagonists for the receptor generally do not penetrate the blood-brain barrier in sufficient quantities for therapeutic action. Noncompetitive antagonists, such as phencyclidine (PCP), have the unwanted properties of abuse liability and propensity to produce psychosis (Zukin and Zukin 1992). Furthermore, neurotoxic actions of PCP, dizocilpine, and related agents, observed when the drugs are administered s.c. to adult rats, limit the development of such drugs for therapeutic purposes (Olney et al. 1989).

Because of the association between activation of the NMDA receptor and the production of NO, it seemed reasonable to propose that NO is an important chemical mediator in opioid dependence or the opioid withdrawal syndrome. The authors hypothesized that NO, as a secondary messenger, may be a mediator of the opioid withdrawal syndrome. If this were true, then inhibitors of NO formation would antagonize opioid withdrawal and could constitute a novel therapeutic approach. Several inhibitors of NOS have been produced by modifying the guanido group of the substrate L-arginine. They include L-N^G-nitroarginine (L-NARG), N^G-monomethyl-L-arginine (NMMA), and L-N^G-nitroarginine methyl ester (L-NAME). To test this hypothesis, the authors measured the effects of inhibitors of NOS on signs of the opioid withdrawal syndrome in morphine-dependent rats.

Experimental subjects for these studies were adult male Fischer-344 rats that were made morphine dependent by the s.c. implantation of morphine pellets. A single dose of 75 mg morphine was administered on the first day of treatment, and two doses of 75 mg were given on the fourth day. Corresponding rats were given s.c. placebo pellets containing the same binder and filler as in the morphine pellets. The morphine treatment reliably produced opioid dependence by the eighth day, when the effects of inhibitors of NOS on opioid withdrawal were tested. Withdrawal was precipitated with an s.c. injection of naloxone (0.5 mg/kg).

L-NARG REDUCES SIGNS OF OPIATE WITHDRAWAL

In the authors' first experiment, L-NARG was administered intraperitoneally (i.p.) twice a day beginning on the fourth day of morphine (or placebo) administration. The dosing schedule was based upon in vitro and in vivo studies indicating that such a regimen produced an irreversible inhibition of NOS in the central nervous system (CNS) (Dwyer et al. 1991). In this preliminary study, 7.5 mg/kg L-NARG tended to reduce the number of wet dog shakes and the amount of weight loss due to diarrhea during precipitated withdrawal (Kimes et al. 1991). Subsequent experiments showed that both the 1 mg/kg and 7.5 mg/kg doses antagonized wet dog shakes (figure l[a]) and that all doses of L-NARG tested (1, 3, and 7.5 mg/kg) in the 4-day, twice-a-day regimen significantly reduced weight loss (figure 1[b]) (Kimes et al. 1993). Neither series of responses exhibited linear regression with dose. L-NARG tended to reduce escape jumps, although the effect was not statistically significant. In addition, 3 mg/kg L-NARG significantly increased teeth chattering in the withdrawing rats. None of the control groups exhibited withdrawal signs after receiving the naloxone challenge.

VARYING THE L-NARG DOSING SCHEDULE

The effect of varying the dosing regimen with L-NARG on efficacy in ameliorating precipitated opioid withdrawal was assessed in another series of experiments (Kimes et al. 1993). Administration of L-NARG by several treatment regimens reduced certain signs of opioid withdrawal. Whether 7.5 mg/kg L-NARG was given i.p. acutely 1 hour before naloxone-precipitated abstinence, twice daily (9 a.m. and 5 p.m.) by the same route on the last 4 days of morphine treatment, or infused continually by s.c.-implanted Alzet osmotic minipumps to deliver 15 mg/kg/day of L-NARG for the entire duration of morphine treatment, the NOS inhibitor significantly antagonized the appearance of wet dog shakes when compared to the appropriate control groups (figures 2[a] and 2[b]). Both the acute and 4-day treatments with L-NARG significantly reduced weight loss that was due primarily to diarrhea, but the 8-day treatment was not effective against this sign of opioid withdrawal (figures 2[c] and 2[d]), perhaps because the chronic treatment induced biosynthesis of peripheral NOS. The observation that L-NARG was effective when given acutely before naloxone-precipitated opioid withdrawal suggested that NO is a mediator of the opioid withdrawal syndrome without necessarily affecting the development of dependence.

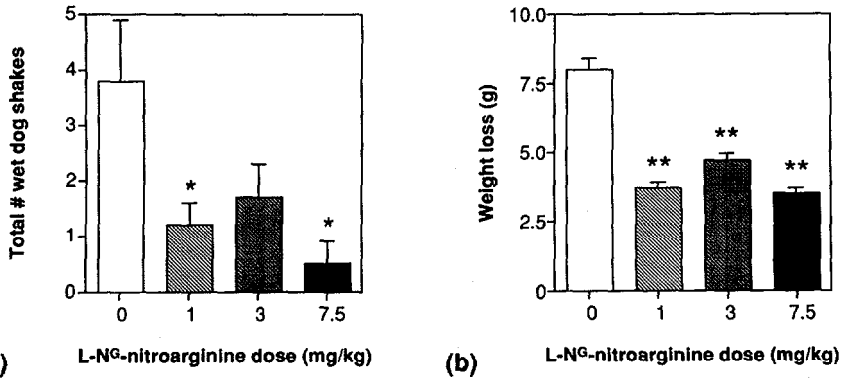

FIGURE 1. *Effect of L-NARG (1, 3, or 7.5 mg/kg, i.p.) on the total number of wet dog shakes (panel a) and on total weight loss (panel b) during the 15 min following precipitation of morphine withdrawal by 0.5 mg/kg naloxone in morphine-dependent rats. Each bar represents the mean±SEM for 8-10 animals, L-NARG was administered twice daily from day 4 to 7 at the doses indicated.*

KEY: * $p < .05$, compared to the saline-treated control group (0 mg/kg L-NARG), Dunnett's test (one-tailed)

DOSE-RESPONSE COMPARISONS OF L-NARG AND L-NAME

A comparison of the potencies of L-NARG and its methyl ester, L-NAME, given acutely 1 hour before the precipitation of abstinence, revealed that both L-NARG and L-NAME reduced wet dog shakes (figures 3[a] and 3[c]) and weight loss (figures 3[b] and 3[d]); they also increased teeth chattering during the first 5 minutes of withdrawal (see figure 4 for results with L-NAME). The methyl ester of L-NARG was less potent than the parent compound in reducing signs of opioid withdrawal; this difference was more obvious in the data on wet dog shakes. Both drugs reduced weight loss and wet dog shakes in a dose-dependent, linear manner. The greater potency of L-NARG was consistent with the activities of the two inhibitors of NOS in vitro (Lambert et al. 1991; Rees et al. 1990), supporting the view that the activities of the drugs in ameliorating opioid withdrawal are due to interactions with NOS.

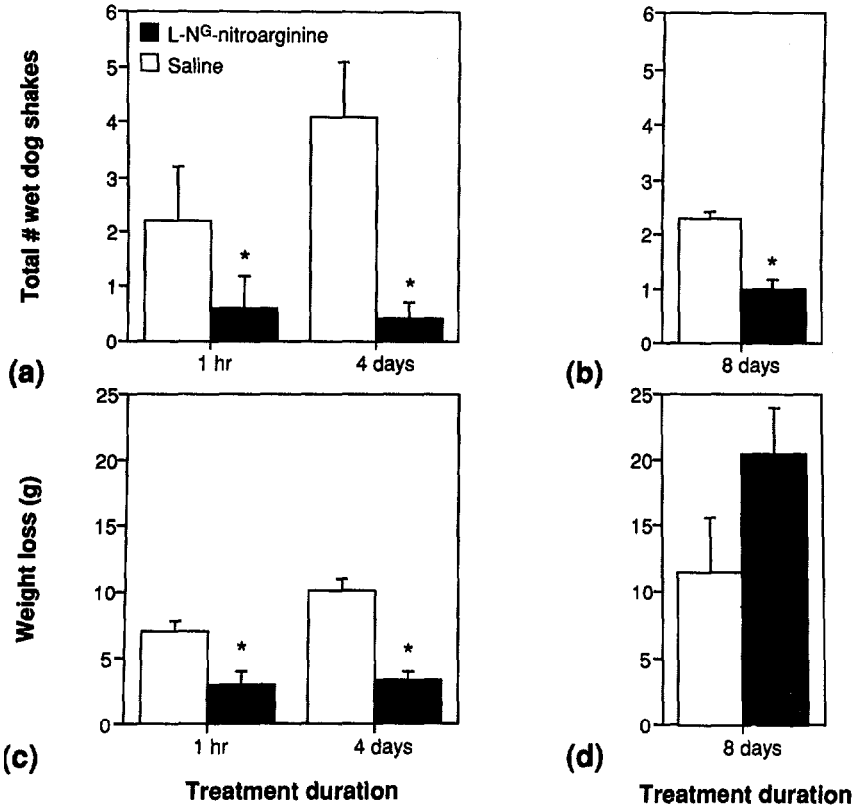

FIGURE 2. *Effect of duration of L-NARG treatment on the total number of wet dog shakes (panels a and b) and total weight loss (panels c and d) in morphine-dependent rats during the 15 min following the precipitation of withdrawal by 0.5 mg/kg naloxone. Data are shown as means±SEM for 6-10 rats/group. The 1-h group received a single injection of saline or L-NARG (7.5 mg/kg, i.p.) 1 h before precipitation, and the I-day groups received injections of saline or L-NARG (7.5 mg/kg, i.p.) twice daily for 4 days before precipitation of abstinence. The S-day groups received continuous infusions of saline or L-NARG (15 mg/kg/day, s.c.) via osmotic pumps implanted at the same time as the first morphine pellet.*

KEY: * $p < .05$, compared to saline-treated controls

The dose dependence observed in the acute treatment study with L-NARG and L-NAME differed from observations in studies of repeated administration of L-NARG (figure 1). When the drug was given twice daily, there was no dose dependency, and there were effects of low doses (1 and 3 mg/kg), suggesting that the drug accumulated with repeated dosing.

A third compound, NMMA, was tested only at one dose, 7.5 mg/kg, administered i.p. 1 hour before precipitation of withdrawal. The only statistically significant effect of this treatment was a reduction in escape jumps.

The spectrum of effects of NOS inhibitors on withdrawal signs in Fischer-344 rats subjected to naloxone-precipitated morphine withdrawal differed from that of clonidine. Clonidine failed to affect teeth chattering, blocked weight loss, reduced wet dog shakes and abnormal posturing, and increased grooming, jumping, and quadrant crossing in rats subjected to naloxone-precipitated morphine withdrawal (Kimes et al. 1990). In contrast, L-NARG and L-NAME reduced wet dog shakes and weight loss and increased teeth chattering (figures 3 and 4). The effect of NMMA was somewhat different. The only effect of this inhibitor of NOS was to reduce the number of escape jumps. The limited effect of NMMA may have been related to an inadequate dosage.

These data suggest that inhibition of NOS primarily affects the manifestation of opioid withdrawal, although they do not exclude a role for NO in the development of dependence in the rat. Assuming that NO production is a consequence of NMDA receptor activation, the authors' data are consistent with the observations that acute antagonism of NMDA receptor activity with dizocilpine and with LY274614 antagonize naltrexone-precipitated morphine withdrawal (Rasmussen et al. 1991*a*). Although dizocilpine prevented the development of dependence in a previous study, acute administration of the NMDA receptor antagonist did not antagonize the appearance of escape jumps in rats subjected to naloxone-precipitated withdrawal, suggesting that NMDA receptor activation was more important in prevention of the development of opioid dependence rather than the expression of the opioid withdrawal syndrome (Trujillo and Akil 1991). However, in the authors' studies, inhibition of NOS is relatively ineffective in antagonizing jumping, as compared with other signs of opioid withdrawal such as wet dog shakes or weight loss. Therefore, it appears that wet dog shakes and weight loss may be more sensitive than other signs to NMDA receptor activation and subsequent

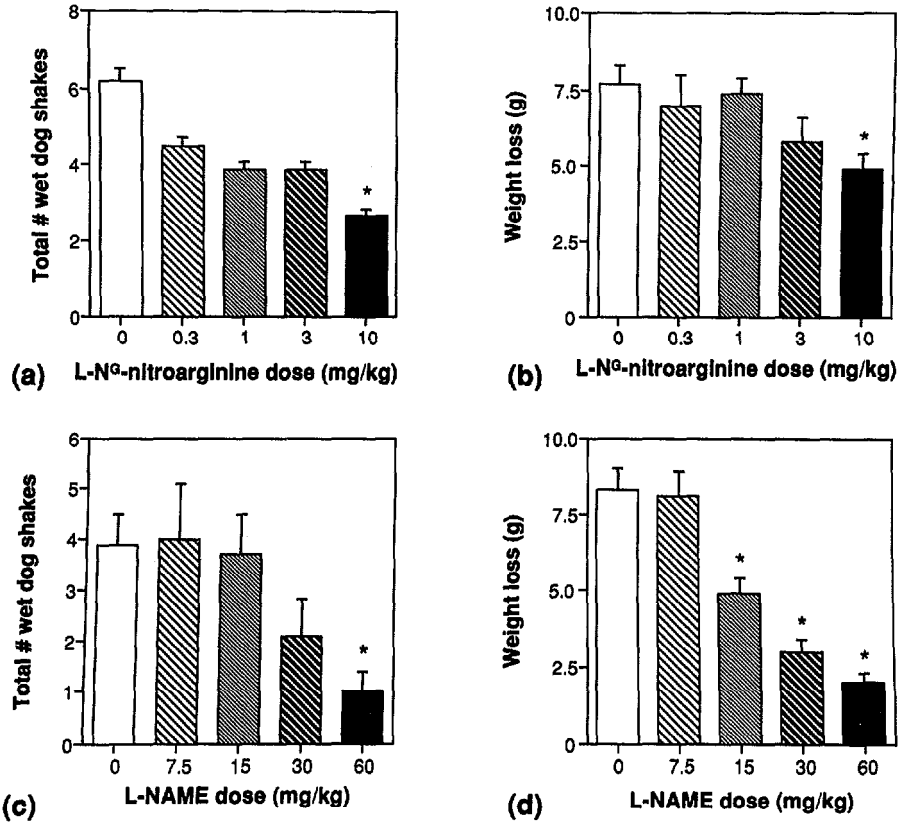

FIGURE 3. *Effects of L-NARG (0, 0.3, 1, 3, or 10 mg/kg, i.p.) and L-NAME (0, 7.5, 15, 30, or 60 mg/kg, i.p.) on the total number of wet dog shakes (panels a and c) and total weight loss (panels b and d) observed in morphine-dependent rats during the 15 min following precipitation of morphine withdrawal by 0.5 mg/kg naloxone. Each bar represents the mean±SEM for 6-10 animals. Drugs were administered as a single injection 1 h before naloxone.*

KEY: * $p < .05$, compared to the saline-treated control group (0 mg/kg drug), Dunnett's test (one-tailed)

production of NO. Furthermore, it seems that inhibition of NOS indeed does prevent the expression of withdrawal in opioid-dependent rats. The authors' results are consistent with findings that repeated administration of NMMA can prevent and reverse opioid dependence in mice given chronic morphine (Kolesnikov et al. 1993).

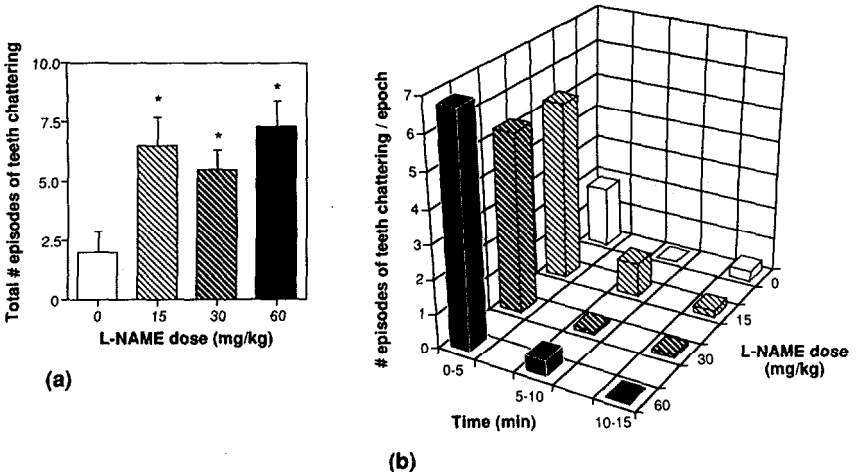

FIGURE 4. *(Panel a) Effects of L-NAME (0, 15, 30, or 60 mg/kg) on the total number of episodes of teeth chattering observed in morphine-dependent rats during the 15 min after naloxone-precipitated withdrawal. Each bar represents the mean±SEM for 6-1 0 animals. L-NAME was administered as a single injection 1 h before the injection of naloxone. (Panel b) Mean number of episodes of teeth chattering in each of three 5-min epochs scored separately.*

KEY: * $p < .05$, compared to the saline-treated control group (0 mg/kg L-NAME) Dunnett's test (one-tailed)

It is of interest to identify the anatomical sites important in the actions of NOS inhibitors on opioid withdrawal. Information on the distribution of glutamatergic pathways and of NOS in the CNS suggests several brain regions that may be important in this regard. The locus coeruleus has been implicated in the integration of opioid actions, including withdrawal (Aghajanian 1978; Cox and Werling 1991). Afferent EAA pathways from the nucleus para gigantocellularis to the locus coeruleus may modulate withdrawal-induced activation of neurons in the locus coeruleus (Rasmussen and Aghajanian 1989). In particular, neurons in the lateral para gigantocellular nucleus contain NOS (Vincent and Kimura 1992). Another important site in opioid withdrawal appears to be the dorsal tegmental nucleus, which shows marked hypermetabolism in rats during naloxone-precipitated morphine withdrawal (Kimes and London 1989; Kimes et al. 1990). Glutamatergic fibers projecting from the laterodorsal tegmental nucleus contain glutamate and NOS (Clements and Grant

1990; Clements et al. 1991; Grant and Highfield 1991). The close proximity of this nucleus to the locus coeruleus suggests that NO in efferents from the laterodorsal tegmental nucleus could act in the locus coeruleus.

The spinal cord also contributes to opioid tolerance and dependence (Bell et al. 1988; Cox and Werling 1991; Martin and Eades 1967). During naloxone-precipitated morphine withdrawal in rats, the substantia gelatinosa exhibits a marked increase in glucose utilization (Bell et al. 1988). Since the substantia gelatinosa contains very high concentrations of NOS (Bredt et al. 1991), hypermetabolism in this region during opioid withdrawal may reflect an increase in NOS activity. In the thoracic region, approximately half of the sympathetic preganglionic neurons forming distinct cholinergic cell clusters in the interomedial lateral cell-column stain for NOS (Calignano et al. 1991). These preganglionic fibers project to the adrenal gland and the coeliac, aorticorenal, and superior mesenteric ganglia. Because autonomic changes constitute part of the abstinence syndrome, NO could be involved in the withdrawal syndrome at this level.

SUMMARY AND CONCLUSION

The NOS inhibitors L-NARG and L-NAME attenuate weight loss and wet dog shakes, two specific signs of opioid withdrawal in rats. NMMA is more potent than L-NAME, consistent with the in vivo actions of these compounds as inhibitors of NOS. In addition, NMMA antagonizes naloxone-induced jumping in morphine-dependent rats. The NOS inhibitors generally increase teeth chattering in precipitated withdrawal. The profile of the diminished withdrawal signs produced by these drugs differs from that produced by clonidine, which stimulates locomotor activity and does not increase teeth chattering in precipitated opioid withdrawal (Kimes et al. 1990). These findings suggest that administration of inhibitors of NOS may be an effective treatment of the opioid withdrawal syndrome alone or in combination with clonidine (US. patent #5,22.5,40).

REFERENCES

Aghajanian, G.K. Tolerance of locus coeruleus neurones to morphine and suppression of withdrawal response by clonidine. Nature 276:186-187, 1978.

Bell, J.A.; Kimes, A.S.; and London, E.D. Increased glucose utilization in superficial layers of the rat spinal dorsal horn during precipitated morphine withdrawal. *Pharmacol Biochem Behav* 150:171-174, 1988.

Bredt, D.S.; Glatt, C.E.; Hwang, P.M.; Fotuhi, M.; Dawson, T.M.; and Snyder, S.H. Nitric oxide synthase protein and mRNA are discretely localized in neuronal populations of the mammalian CNS together with NADPH diaphorase. Neuron 7:615-624, 1991.

Calignano, A.; Moncada, S.; and Dirosa, M. Endogenous nitric oxide modulates morphine-induced constipation. *Biochem Biophys Res Commun* 181:889-893, 199l.

Clements, J.R., and Grant, S. Glutamate-like immunoreactivity in neurons of the laterodorsal tegmental and pedunculopontine nuclei in the rat. *Neurosci Lett* 120:70-73, 1990.

Clements, J.R.; Toth, D.D.; Highfield, D.A.; and Grant, S.J. Glutamate-like immunoreactivity is present within cholinergic neurons of the laterodorsal tegmental and pedunculopontine nuclei. In: Napier, T.C., ed. *The Basal Forebrain.* New York: Plenum Press, 1991. pp. 127-142.

Cox, B.M., and Werling, L.L. Opioid tolerance and dependence. In: Pratt, J.A., ed. *The Biological Bases of Drug Tolerance and Dependence.* New York: Academic Press, 1991. pp. 199-229.

Dwyer, M.A.; Bredt, D.S.; and Snyder, S.H. Nitric oxide synthase: Irreversible inhibition by L-N^G-nitroarginine in brain *in vitro* and *in vivo. Biochem Biophys Res Commun* 176:1136-1141, 1991.

Garthwaite, J. Glutamate, nitric oxide and cell-cell signalling in the nervous system. *Trends Neurosci* 14:60-67, 1991.

Garthwaite, J.; Charles, S.L.; and Chess-Williams, R. Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. *Nature* 366:385-388, 1988.

Grant, S.J., and Highfield, D.A. Extracellular characteristics of putative cholinergic neurons in the rat laterodorsal tegmental nucleus. *Brain Res* 559:64-74, 1991.

Kimes, A.S.; Bell, J.A.; and London, E.D. Clonidine attenuates increased brain glucose metabolism during naloxone-precipitated morphine withdrawal. *Neuroscience* 34:633-644, 1990.

Kimes, A.S., and London, E.D. Glucose utilization in the rat brain during chronic morphine treatment and naloxone-precipitated morphine withdrawal. *J Pharmacol Exp Ther* 248:538-545, 1989.

Kimes, A.S.; Vaupel, D.B.; Bruckner, M.; and London, E.D. Nitroarginine, a nitric oxide synthase inhibitor, attenuates morphine withdrawal. *Soc Neurosci Abstr* 17:538, 1991.

Kimes, A.S.; Vaupel, D.B.; and London, E.D. Attenuation of some signs of opioid withdrawal by inhibitors of nitric oxide synthase. *Psychopharmacology (Berl)* 45:521-524, 1993.

Kolesnikov, Y.A.; Pick, C.G.; Ciszewska, G.; and Pasternak, G.W. Blockade of tolerance to morphine but not to κ opioids by a nitric oxide synthase inhibitor. *Proc Natl Acad Sci U S A* 90:5162-5166, 1993.

Lambert, L.E.; Whitten, J.P.; Baron, B.M.; Cheng, H.C.; Doherty, N.S.; and McDonald, I.A. Nitric oxide synthesis in the CNS endothelium and macrophages differs in its sensitivity to inhibition by arginine analogues. *Life Sci* 48:69-75, 1991.

Martin, W.R., and Eades, C.G. Pharmacological studies of spinal cord adrenergic and cholinergic mechanisms and their relation to physical dependence on morphine. *Psychopharmacology (Berl)* 11: 195-223, 1967.

Olney, J.W.; Labruyere, J.; and Price, M.T. Pathological changes induced in cerebrocortical neurons by phencyclidine and related drugs. *Science* 244:1360-1362, 1989.

Rasmussen, K., and Aghajanian, G.K. Withdrawal-induced activation of locus coeruleus neurons in opiate-dependent rats: Attenuation by lesions of the nucleus paragigantocellularis. *Brain Res* 505:346-350, 1989.

Rasmussen, K.; Fuller, R.W.; Stockton, M.E.; Perry, K.W.; Swinford, R.M.; and Omstein, P.L. NMDA receptor antagonists suppress behaviors but not norepinephrine turnover or locus coeruleus unit activity induced by opiate withdrawal. *Eur J Pharmacol* 197:9-16, 1991*a*.

Rasmussen, K.; Krystal, J.H.; and Aghajanian, G.K. Excitatory amino acids and morphine withdrawal: Differential effects of central and peripheral kynurenic acid administration. *Psychopharmacology (Berl)* 105:508-512, 1991*b*.

Rees, D.D.; Palmer, R.M.; Schulz, R.; Hodson, H.F.; and Moncada, S. Characterization of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. *Br J Pharmacol* 101:746-752, 1990.

Trujillo, K.A., and Akil, H. Inhibition of morphine tolerance and dependence by the NMDA receptor antagonist MK-801. *Science* 251:85-87, 1991.

Vincent, S.R., and Kimura, H. Histochemical mapping of nitric oxide synthase in the rat brain. *Neuroscience* 46:755-784, 1992.

Zukin, S.R., and Zukin, R.S. Phencyclidine. In: Lowinson, J.H.; Ruiz, P.; Millman, R.B.; and Langrod, J.G., eds. *Substance Abuse: A Comprehensive Textbook.* Baltimore: Williams & Wilkins, 1992. pp. 290-302.

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Nitric Oxide and Opioid Tolerance

Gavril W. Pasternak

INTRODUCTION

Tolerance is easily defined as a diminishing response to a constant dose of drug or the need to increase a drug dosage to maintain a constant pharmacological response. This phenomenon invariably accompanies chronic opioid use. Tolerance has proven complicated and difficult to understand, with evidence supporting several potential mechanisms. Tolerance can be produced at the level of the receptor, its transduction mechanisms, the cell, or through complex interactions with other neurotransmitter systems with the cloning of several opioid receptors (Chen et al. 1994; Evans et al. 1992; Fukuda et al. 1993; Kieffer et al. 1992; Yasuda et al. 1993); investigations of potential molecular changes are just beginning. Studies have suggested changes in the numbers of binding sites with tolerance (Danks et al. 1988; Lahti and Collins 1978; Law et al. 1983). Others have implicated transduction systems (Blume et al. 1979; Costa et al. 1988, 1992; Frey and Kebabian 1984; Kennedy and Henderson 1991; McKenzie and Milligan 1990; Puttfarcken et al. 1988; Sharma et al. 1975; Smith et al. 1988). Chronic opioid exposure of NG108-15 cells up-regulates adenylyl cyclase and is associated with a decreased inhibition of cyclic AMP (cAMP) synthesis by opioids (Law et al. 1983). Presumably, the increased enzyme levels compensate for the inhibitory actions of the opioids and restore homeostasis by returning cAMP levels to normal levels. Withdrawal of the opioids or administration of an antagonist removes the inhibitory actions of the opioids, resulting in a rebound elevation of cAMP levels, possibly explaining dependence.

Despite evidence supporting a receptor or second messenger mechanism in tissue culture, it is not clear that this is the primary mechanism in vivo (Pastemak 1993). It is equally possible that tolerance results from the activation of compensatory antagonistic systems within the brain. A variety of opioid systems can modulate opioid analgesia (Botney and Fields 1983; Dourish et al. 1988; Faris et al. 1983; Pick et al. 1992; Watkins et al. 1984). Cholecystokinin (CCK) administered into the periaqueductal gray markedly diminishes the analgesic effects of morphine. On the other hand, many compounds enhance opioid analgesia, including antidepressants and CCK antagonists like

proglumide and a number of antidepressants. All these systems act in both tolerant and naive animals, implying a generalized potentiation of opioid action rather than a specific effect on tolerance.

N-methyl-D-Aspartate (NMDA) RECEPTORS AND TOLERANCE

Disinguishing between simple potentiation of opioid action and a selective effect on tolerance is important. Recently, Trujillo and Akil (1991) reported that the noncompetitive NMDA antagonist MK-801 prevents morphine tolerance. Furthermore, MK-801 reverses preestablished morphine tolerance and reduces dependence. At the same time, MK-801 does not affect morphine's analgesic potency in opioid-naive animals, implying a specific action on tolerance. The action is not restricted to noncompetitive antagonists. Competitive NMDA antagonists acting at a different location on the receptor also prevent tolerance to morphine and reverse established tolerance despite continued administration of morphine (Elliot et al. 1994; Kolesnikov et al. 1993*a*; Tiseo et al. 1994). Recent work also has indicated that a competitive antagonist for the glycine site on the NMDA receptor, 1-amino-cyclopropane carboxylic acid (ACPC), interferes with morphine tolerance. Thus, three classes of drugs acting on NMDA receptors modulate opioid tolerance.

NITRIC OXIDE (NO) AND TOLERANCE

Many NMDA actions are linked closely to the activation of nitric oxide synthase (NOS), the enzyme which generates NO (Bredt and Snyder 1992; Garthwaite et al. 1988). NO is an unusual neurotransmitter. As a gas, NO cannot be stored within vessicles like other neurotransmitters and, thus, must be manufactured upon demand. Thus, activation and inhibition of NOS provides relatively rapid changes in neurotransmission. In view of the dramatic effects of the NMDA-related compounds, studies were initiated on the effects of N^G-nitro-L-arginine (NOArg), an inhibitor of NOS (Babey et al., in press; Kolesnikov et al. 1992, 1993*a*). Morphine pellet implantation induces the rapid development of tolerance, which is attenuated significantly by NOArg (figure 1). Pellet implantation has a number of disadvantages. Pellets quickly release very high morphine doses, with virtually all animals exceeding the maximal analgesic cutoffs. This type of dosing does not mimic the clinical situation, where patients initially receive lower doses of analgesics that

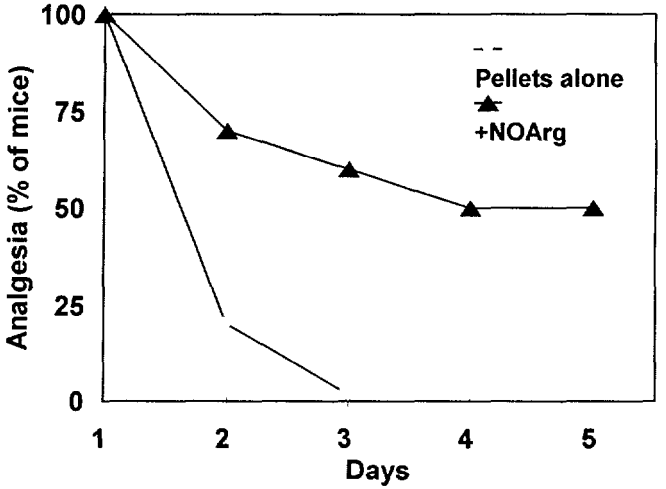

FIGURE 1. *Effects of NOArg on tolerance to morphine pellets. Groups of mice were implanted with morphine pellets (75 mg, freebase) on day 1 and treated with NOArg or nothing. Analgesia was assessed quantally as a doubling or greater in the tail flick assay.*

SOURCE: Kolesnikov et al. (1993*a*)

then are escalated. Furthermore, few compounds are available in pellet form, greatly limiting the number and type of opioids that can be examined, particularly the opioid peptides. In view of this difficulty, a different paradigm was developed to examine tolerance.

The opioid was administered once daily at a dose sufficient to elicit analgesia in approximately 50-70 percent of mice on the first day, making it a clinically relevant dose. This dosing schedule produces a slow but steady decrease in morphine sensitivity (figure 2). After 5 days, morphine's ED, doubles, and by 10 days it increases approximately fourfold. Extending the dosing to 28 days further increases the ED,.

In this paradigm, NOArg prevents tolerance to morphine (figure 3[a]). The activity of the 2 mg/kg/day dose is maintained for at least 28 days, the longest time tested. These observations were confirmed by full morphine dose-response curves after 5 and 10 days. In contrast to results

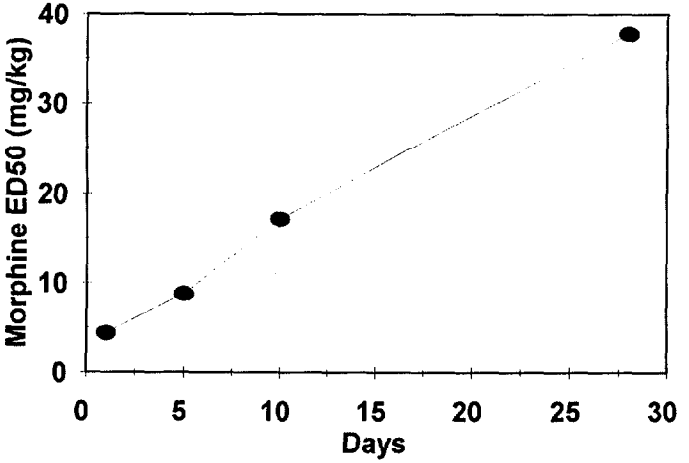

FIGURE 2. *ED, changes with daily morphine dosing. Groups of mice received morphine (5 mg/kg, subcutaneously [s.c.]) daily for the indicated number of days, and then ED, values were determined from dose-response curves. Each point represents a different group of mice.*

SOURCE: Kolesnikov et al. (1993*a*)

with morphine alone, co-administration of NOArg prevents any significant change in the ED, values.

To assess whether these actions of NOArg involved mechanisms of tolerance, its actions in naive mice also were examined. The similar response to the NOArg and control mice on the first day suggested that NOArg has no effect on analgesia in naive animals. This observation was confirmed with full morphine ED, determinations (Kolesnikov et al. 1993*a*). Even pretreatment of the mice with NOArg for 5 days prior to testing with morphine does not affect morphine sensitiviiy. Thus, the actions of NOArg are limited to tolerance.

REVERSAL OF MORPHINE TOLERANCE

Next, it was explored whether or not NOArg could reverse preexisting tolerance. Morphine only was administered daily for 5 days. The animals then were divided, with one group continuing to receive

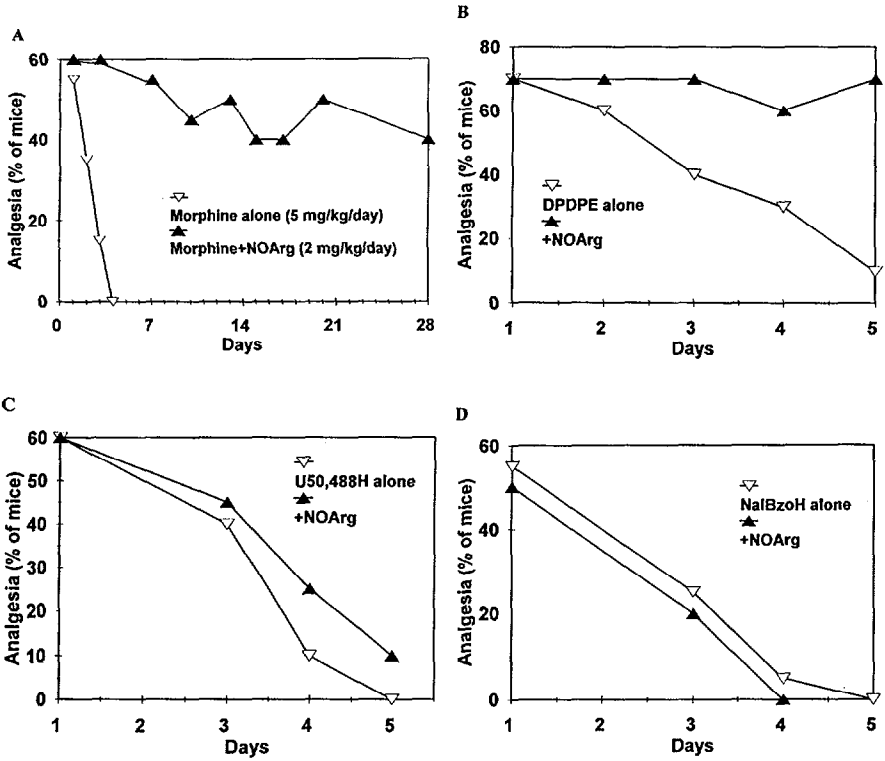

FIGURE 3. *Effects of NOArg on morphine, DPDPE, U-50,488H, and naloxone benzoylhydrazone (NalBzoH) analgesia. Groups of mice received morphine (s.c.), DPDPE (intraperitoneally), U-50,488H (s.c.) or NalBzoH (s.c.) daily along with NOArg or nothing.*

SOURCE: Kolesnikov et al. (1993*a*)

morphine alone and the other morphine with NOArg. As indicated earlier, continuing morphine dosing from 5 days to 10 days further increases the ED, of morphine by approximately twofold. Co-administration of NOArg along with morphine from days 5 through 10 shows a return of analgesic sensitivity despite the continued morphine dosing (figure 4). Indeed, the analgesic response with the combination of morphine and NOArg together exceeds that seen by abstinence (Kolesnikov et al. 1993*a*). Thus, NOArg reverses preexisting morphine tolerance.

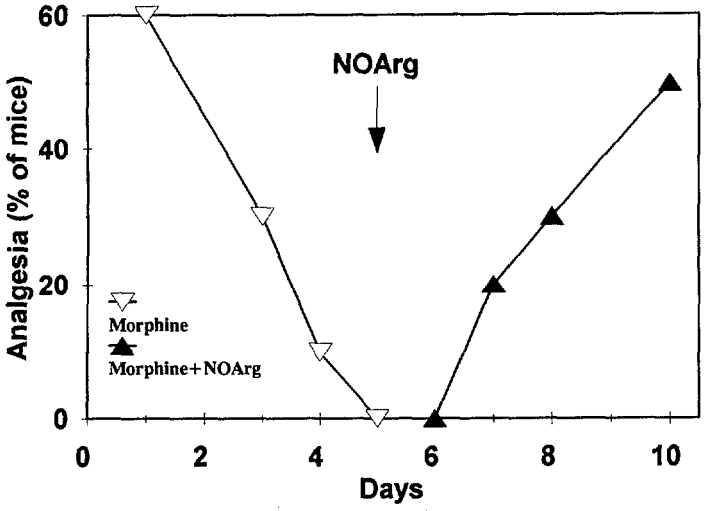

FIGURE 4. *Reversal of morphine tolerance by NOArg. Mice received morphine alone for 5 days, followed by morphine along with NOArg from days 6 through 10.*

SOURCE: Kolesnikov et al. (1993*a*)

The specificity of NOArg's actions is demonstrated further by studies with arginine. L-arginine is the natural substrate for the enzyme NOS. L-arginine, given acutely, markedly reduces the analgesic activity of morphine, and chronic administration enhances the development of tolerance. Furthermore, L-arginine blocks NOArg's actions on morphine tolerance (i.e., when L-arginine and NOArg are given together, tolerance to morphine develops quite rapidly). L-arginine is without effect.

NO AND DEPENDENCE

Although analgesic tolerance often is associated with dependence, evidence from the Memorial Sloan-Kettering Cancer Center has indicated the importance of different receptor subtypes in the two actions (Ling et al. 1984). Previous work had indicated that NOArg blocks the development of dependence (Kimes et al. 1991). This result was confirmed and extended to demonstrate that NOArg could reverse preexisting dependence as well.

NOArg prevents tolerance through the inhibition of the enzyme NOS. Nitrite formation, an indication of NO activity, is reduced markedly by NOArg. Furthermore, NOS activity measured in vitro is antagonized by NOArg given in vivo at the doses active against tolerance. However, chronic morphine administration does not change the levels of enzymatic activity, nor does it change the levels of messenger RNA coding for the constitutive NOS enzyme (Babey et al., in press). Thus, opiates appear to activate the enzyme without changing its expression.

NO AND DELTA TOLERANCE

Delta receptors modulate pain perception, particularly at the spinal level (Pasternak 1993; Porreca et al. 1987; Yaksh 1985). Extending the daily injection paradigm described with morphine to an intrathecal approach, it can be observed that daily administration of DPDPE enkephalin slowly produces tolerance with approximately the same time course as morphine (figure 3[b]). With a fixed daily dose, analgesia declines from approximately 50-70 percent of mice on day 1 to 10 percent or less by day 5. NOArg (2 mg/kg) blocks this tolerance. Similarly, MK-801, a number of competitive NMDA antagonists, and the glycine antagonist ACPC also prevent tolernace. This common response to mu and delta analgesic tolerance is quite intriguing since the two systems do not show cross-tolerance to each other.

NO AND KAPPA ANALGESIA

Kappa receptors represent yet another class of opioid receptors (Martin et al. 1976; Cheng et al. 1992; Clark et al. 1989; Gistrak et al. 1989; Kosterlitz et al. 1981; Paul et al. 1990*a*, 1990*b*, 1991; Rothman et al. 1990; Standifer et al., in press; Zukin et al. 1988). Tolerance to the analgesic actions of U-50,488H (Von Voightlander et al. 1983), a kappa, analgesic, was examined next. Tolerance to U-50,488H is unaffected by MK-801, a number of competitive NMDA antagonists, and NOArg at doses as high as 8 mg/kg (figure 3[c]). However, one competitive NMDA antagonist, NPC17742, effectively blocks U-50,488H tolerance (Kolesnikov et al. 1993*b*). This activity is quite interesting since it differs so dramatically from the other NMDA antagonists. Several possibilities exist. Either NPC17742 has a receptor selectivity distinct from the other NMDA receptors, or it interacts with the receptor in a novel way. It is intriguing to contemplate the possibility of NMDA subtypes that can be

distinguished by NMDA antagonists. However, these issues remain speculative and will require further investigation.

Daily administration of the kappa, analgesic naloxone benzoylhydrazone (NalBzoH) (Gistrak et al. 1989; Paul et al. 1990*a*) produces tolerance, with the analgesic response gradually declining from 60 percent down to 0 percent over the course of 5 days (figure 3[d]). Unlike mu and delta tolerance, the tolerance seen with NalBzoH is not affected by MK-801, the competitive NMDA antagonists, or NOArg. Furthermore, NPC17742 does not influence NalBzoH tolerance, providing an important distinction between kappa, and kappa, analgesic mechanisms and their tolerance.

CONCLUSIONS

Tolerance is a complex series of events and almost certainly is mediated through a number of mechanisms. Tolerance can be observed at the level of the receptor or the cell, as well as by interactions with other neurotransmitter systems. It would be inappropriate to try to define a single mechanism for the actions of opiates in vivo. However, it is important to try to understand the mechanism most relevant to the clinical setting. These studies suggest that the activation of compensatory antagonistic systems is the predominant mechanism of morphine tolerance in vivo with moderate analgesic doses. The initial studies with MK-801 now have been extended to a variety of competitive NMDA antagonists, as well as blockers of the glycine site on the NMDA receptor. These, in turn, have led to observations implicating NO as a potential second step in the cascade leading to tolerance. However, the formation of NO almost certainly is not the final step. If it were, inhibition of NOS would have been expected to immediately reverse preexisting tolerance. This does not occur. Rather, analgesic sensitivity returns over approximately 5 days, implying the slow decay of the systems responsible for tolerance. These observations imply that the existence of steps beyond NO that are important in the final production of tolerance. Many questions remain, including the target of NO.

In conclusion, morphine tolerance can be blocked without interfering with analgesia. The implication of these studies is important. Although tolerance oftentimes can be offset by increasing doses of drugs, this option frequently is limited by the appearance of side effects. Not all actions of opioids produce tolerance at the same rate or to the same degree (Ling et al. 1989). For example, tolerance to gastrointestinal

actions and respiratory depression develop more slowly than tolerance to analgesia. Increasing drug doses, therefore, is associated with a lower therapeutic index and the potential of greater problems with side effects at the same level of analgesia. Furthermore, tolerance requires constant adjustments of drug doses, making it even more difficult for physicians to treat patients adequately. Being able to administer opiates without the fear of significant tolerance or dependence may provide significant advantages in the use of opioid analgesics.

ACKNOWLEDGMENT

This work was supported, in part, by grants from the National Institute on Drug Abuse (DA07242 and DA000138) to Dr. Pastemak.

REFERENCES

Babey, A.M.; Kolesnikov, Y.A.; Cheng, J.; Inturrisi, C.E.; Trifilletti, R.R.; and Pastemak, G.W. Nitric oxide and opioid tolerance. *Neurophamacology,* in press.

Blume, A.J.; Lichtstein, D.; and Boone, G. Coupling of opiate receptors to adenylate cyclase: Requirements for Na^+ and GTP. *Proc Natl Acd Sci U S A* 76:5626-5630, 1979.

Botney, M., and Fields, H.L. Amitriptyline potentiates morphine analgesia by a direct action on the central nervous system. *Ann Neurol* 13:160-164, 1983.

Bredt, D.S., and Snyder, S.H. Nitric oxide, a novel neuronal messenger. *Neuron* 8:3-11, 1992.

Chen, Y.; Mestek, A.; Liu, J.; Hurley, J.A.; and Yu, L. Molecular cloning and functional expression of a mu opioid receptor from rat brain. Mol *Pharmacol* 44:8-12, 1994.

Cheng, J.; Rocques, B.P.; Gacel, G.A.; Huang, E.; and Pastemak, G. W. Kappa, opiate receptor binding in the mouse and rat. *Eur J Pharmacol* 226:15-20, 1992.

Clark, J.A.; Liu, L.; Price, M.; Hersh, B.; Edelson, M.; and Pastemak, G.W. Kappa receptor multiplicity: Evidence for two U50,488-sensitive κ_1 subtypes and a novel κ_3 subtype. *J Pharmacol Exp Ther* 251:461-468, 1989.

Costa, E.M.; Hoffman, B.B.; and Loew, G.H. Opioid agonists binding and responses in SH-SY5Y cells. *Life Sci* 50:73-81, 1992.

Costa, T.; Klinz, F.-J.; Vachon, L.; and Herz, A. Opioid receptors are coupled tightly to G proteins but loosely to adenylate cyclase in NG1O8-15 cell membranes. *Mol Pharmacol* 34:744-754, 1988.

Danks, J.A.; Tortella, F.C.; Long, J.B.; Bykov, V.; Jacobson, A.E.; Rice, K.W.; Holaday, J.W.; and Rothman, R.B. Chronic administration of morphine and naltrexone up-regulates [^{3}H][D-ala^2, D-leu^5] enkephalin binding sites by different mechanisms. *Neurophamacology* 27: 965-974, 1988.

Dourish, C.T.; Hawley, D.; and Iversen, S.D. Enhancement of morphine analgesia and prevention of morphine tolerance in the rat by the CCK antagonist, L-364,718. *Eur J Pharmacol* 147:469, 1988.

Elliot, C.; Minami, N.; Kolesnikov, Y.; Pastemak, G.W.; and Inturrisi, C.E. The NMDA antagonists, LY274614 and MK801, and the nitric oxide synthase inhibitor, N^G-nitro-L-arginine attenuate the analgesic tolerance to the mu opioid morphine, but not to kappa opioids. *Pain* 56:69-75, 1994.

Evans, C. J.; Keith, D.E.; Morrison, H.; Magendzo, K.; and Edwards, R.H. Cloning of a delta opioid receptor by functional expression. *Science* 258:1952-1955, 1992.

Faris, P.L.; Komisaruk, B.R.; Watkins, L.R.; and Mayer, D.L. Evidence for the neuropeptide cholecystokinin as an antagonist of opiate analgesia. *Science* 219: 310-312, 1983.

Frey, E.A., and Kebabian J.W. A p-opiate receptor in 7315c tumor tissue mediates inhibition of immunoreactive prolactin release and adenylate cyclase activity. *Endocrinology* 115:1797-1804, 1984.

Fukuda, K.; Katto, S.; Mori, K.; Nishi, M.; and Takeshima, H. Primary structures and expression from cDNAs of rat opioid receptor δ-and μ-subtypes. *FEBS Lett* 327:311-314, 1993.

Garthwaite, J.; Charles, S.L.; and Chess-Williams, R. Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. *Nature* 336:385-388, 1988.

Gistrak, M.A.; Paul, D.; Hahn, E.F.; and Pasternak, G.W. Pharmacological actions of a novel mixed agonist-antagonist: naloxone benzoylhydrazone. *J Pharmacol Exp Ther* 251:469-476, 1989.

Kennedy, C., and Henderson, G. μ-opioid receptor inhibition of calcium current: Development of homologous tolerance in single SH-SY5Y cells after chronic exposure to morphine *in vitro*. *Mol Pharmacol* 40:1000-1005, 1991.

Kieffer, B.L.; Befort, K.; Gaveriaux-Ruff, C.; and Hirth, C.G., The δ opioid receptor: Isolation of a cDNA by expression cloning and pharmacological characterization. *Proc Natl Acad Sci U S A* 89:12048-12052, 1992.

Kimes, A.S.; Vaupel, D.B.; Bruckner, M.; and London, E.D. Nitroarginine, a nitric oxide synthase inhibitor, attenuates morphine withdrawal. Soc *Neurosci* 17:214.12, 1991.

Kolesnikov, Y.; Ferkany, J.; and Pastemak, G.W. Blockade of mu and kappa, opioid analgesic tolerance by NPC17742, a novel NMDA antagonist. *Life Sci* 53:1489-1494, 1993*b*.

Kolesnikov, Y.; Pick, C.G.; Ciszewska, G.; and Pastemak, G.W. Blockade of tolerance to morphine but not to kappa opioids by a nitric oxide synthase inhibitor. *Proc Natl Acad Sci U S A* 90:5162-5166, 1993*a*.

Kolesnikov, Y.; Pick, C.G.; and Pastemak, G.W.N^G-Nitro-L-Arginine prevents morphine tolerance. *Eur J Pharmacol* 221:399-400, 1992.

Kosterlitz, H.W.; Paterson, S.J.; and Robson, L.E. Characterization of the kappa subtype of the opiate receptor in the guinea pig brain. *Br J Phamacol* 68:333-342, 1981.

Lahti, R.A., and Collins, K.J. Chronic naloxone results in prolonged increases in opiate binding sites in brain. *Eur J Pharmacol* 51:185-186, 1978.

Law, P.Y.; Horn, D.S.; and Loh, H.H. Opiate receptor down-regulation and desensitization in neuroblastoma X glioma NG108-15 hybrid cells are two separate cellular adaptation processes. *Mol Pharmacol* 25:413-424, 1983.

Ling, G.S.F.; Macleod, J.M.; Lee, S.; Lockhart, S.; Pastemak, G.W. Separation of morphine analgesia from physical dependence. *Science* 226:462-464, 1984.

Ling, G.S.F.; Paul, D.; Simantov, R.; Pastemak, G.W. Differential development of acute tolerance to analgesia, respiratory depression, gastrointestinal transit and hormone release in a morphine infusion model. *Life Sci* 45:1627-1636, 1989.

Martin, W.R.; Eades, C.G.; Thompson, J.A.; Huppler, R.E.; and Gilbert, P.E. The effects of morphine and nalorphine-like drugs in the nondependent and morphine-dependent chronic spinal dog. *J Pharmacol Exp Ther* 197:517-532, 1976.

McKenzie, F.R., and Milligan, G. δ--Opioid-receptor-mediated inhibition of adenylate cyclase is transduced specifically by the guanine-nucleotide-binding protein G_j2. *Biochem J* 267:391-398, 1990.

Pastemak, G. Pharmacological mechanisms of opioid analgesics. *Clin Neurophamacol* 16:1-18, 1993.

Paul, D.; Levison, J.A.; Howard, D.H.; Pick, C.G.; Hahn, E.F.; and Pastemak, G.W. Naloxone benzoylhydrazone (NalBzoH) analgesia. *J Pharmacol Exp Ther* 255:769-774, 1990*a*.

Paul, D.; Pick, C.G.; Tive, L.A.; and Pastemak, G.W. Pharmacological characterization of nalorphine, a kappa, analgesic. *J Pharmacol Exp Ther* 255:769-774, 1990*b*.

Paul, D.; Pick, C.G.; Tive, L.A.; and Pastemak, G.W. Pharmacological characterization of nalorphine, a κ_3 analgesic. *J Pharmacol Exp Ther* 257:1-7, 1991.

Pick, C.G.; Paul, D.; Eison, M.S.; and Pastemak, G.W. Potentiation of opioid analgesia by the antidepressant Nefazadone. *Eur J Phannacol* 211:375-381, 1992.

Porreca, F.; Heyman, J.S.; Mosberg, HI.; Omnaas, F.R.; and Vaught, J.L. Role of mu and delta receptors in the supra spinal and spinal analgesic effects of [D-Pen^2, D-Pen^5] lenkephalin in the mouse. *J Pharmzcol Exp Ther* 24:393-400, 1987.

Puttfarcken, P.; Werling, L.L.; and Cox, B.M. Effects of chronic morphine exposure on opioid inhibition of adenylyl cyclase in 7315c cell membranes: A useful model for the study of tolerance at μ opioid receptors. *Mol Pharmacol* 33:520-527, 1988.

Rothman, R.B.; Bykov, V.; DeCosta, B.R.; Jacobson, A.E.; Rice, K.C.; and Brady, L.S. Interaction of endogenous opioid peptides and other drugs with four kappa opioid binding sites in guinea pig brain. *Peptides* 11:311-331, 1990.

Sharma, S.K.; Klee, W.A.; and Nirenberg, M. Dual regulation of adenylate cyclase accounts for narcotic dependence and tolerance. *Proc Natl Acad Sci U S A* 72:3092-3096, 1975.

Smith, A.P.; Law, P.Y.; and Loh, H.H. Role of opioid receptors in narcotic tolerance/dependence. In: Pastemak, G.W., ed. *The Opiate Receptors*. Clifton, NJ: The Humana Press, 1988. pp. 441-485.

Standifer, K.M.; Cheng, J.; Brooks, A.I.; Honrado, C.P.; Su, W.; Visconti, L.M.; Biedler, J.L.; and Pastemak, G.W. Biochemical and pharmacological characterization of mu, delta and kappa, opioid receptors expressed in BE(2)-C neuroblastoma cells. *J Pharmacol Exp Ther,* in press.

Tiseo, P.J.; Cheng, J.; Pastemak, G.W.; and Inturrisi, C.E. Modulation of morphine tolerance by the Competitive NMDA receptor antagonist LY274614: Assessment of opioid receptor changes. *J Pharmacol Exp Ther* 268:195-201, 1994.

Trujillo, K.A., and Akil, H. Inhibition of morphine tolerance and dependence by the NMDA receptor antagonist MK-801. *Science* 251:85-87, 1991.

Von Voightlander, P.F.; Lahti, R.A.; and Ludens, J. U50,488: A selective and structurally novel non-mu (kappa) opioid agonist. *J Pharmacol Exp Ther* 224:7-12, 1983.

Watkins, L.R.; Kinscheck, I.B.; and Mayer, D.J. Potentiation of opiate analgesia and apparent reversal of morphine tolerance by proglumide, a cholecystokinin antagonist. *Science* 224:395-399, 1984.

Yaksh, T.L. The physiology and pharmacology of spinal opiates. Annu *Rev Pharmacol Toxicol* 25-433-462, 1985.

Yasuda, K.; Raynor, K.; Kong, H.; Breder, C.D.; Takeda, J.; Reisine, T.; and Bell, G.I. Cloning and functional expression of κ and δ opioid receptors from mouse brain. *Proc Natl Acad Sci U S A* 90:6736-6740, 1993.

Zukin, R.S.; Eghbali, M.; Olive, D.; Unterwald, E.M.; and Tempel, A. Characterization and visualization of rat and guinea pig brain κ opioid receptors: Evidence for k_1, and k_2 opioid receptors. *Proc Natl Acad Sci U S A* 85:4061-4065, 1988.

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Methoclocinnamox: A μ Partial Agonist With Pharmacotherapeutic Potential for Heroin Abuse

James H. Woods, John W. Lewis, Gail Winger, Eduardo Butelman, Jillian Broadbear, and Gerald Zernig

INTRODUCTION

Methoclocinnamox [14ß-(p-chlorocinnamoylamino)-7, 8-dihydro-N-cyclopropylmethyl norcodeinone mesylate] (see figure 1[a]) is a member of an interesting set of 14-substituted codeinones and morphinones that have yielded opioid agonists, partial agonists, and antagonists with selectivity of action at the μt receptor (Lewis et al. 1988). Methoclocinnamox is converted metabolically to clocinnamox (figure 1[b]), a very long-acting μ receptor antagonist (Aceto et al. 1989; Comer et al. 1992). Methoclocinnamox's N-substituent, viz., cyclopropylmethyl, probably confers low efficacy at opioid receptors. The cinnamoylamino chain at the 14 position confers a weak Michael acceptor property, thus providing the possibility of irreversibility at opioid receptors. Methoclocinnamox is more difficult to wash from brain membrane binding preparations than reversible agonists (Zemig, unpublished observations).

This communication will summarize the pharmacological properties of methoclocinnamox, both in vitro and in vivo, that suggest that the compound may be of considerable interest as a therapeutic agent for heroin (or other opioid) abuse. It has some functional characteristics that make it similar to buprenorphine, and the reader should have this comparison in mind when considering the material to be presented. Attention will be drawn to the similarities of methoclocinnamox, clocinnamox, and buprenorphine in the various assays in order to display some of their more important characteristics.

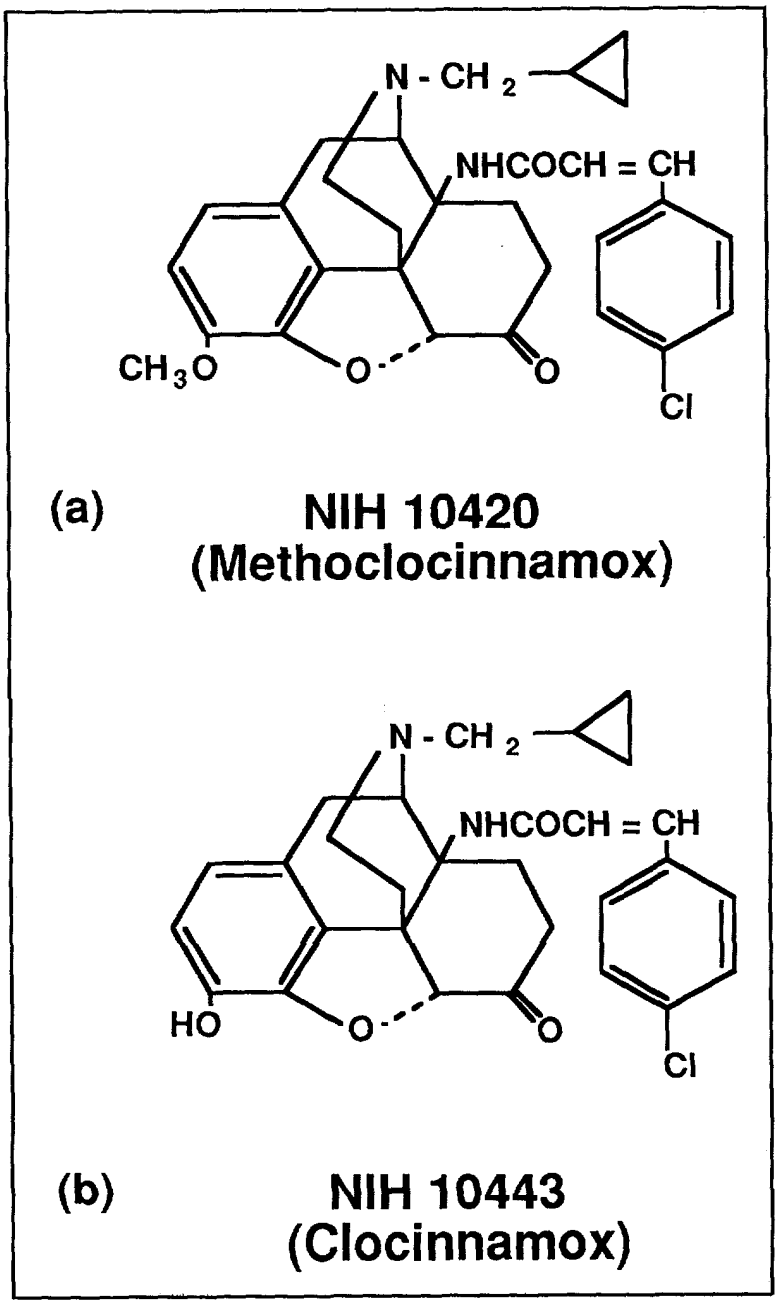

FIGURE 1. *Molecular structures of methoclocinnamox and of clocinnamox*

IN VITRO PREPARATIONS

Opioid Receptor Binding

Methoclocinnamox, clocinnamox, and buprenorphine have high affinity for tritiated etorphine binding sites with IC_{50}s of 1.0, 0.6, and 1.2nM, respectively (Aceto et al. 1989; G. Zernig, unpublished observations). Morphine's IC_{50} is 23 nM. Recently, Burke and colleagues (1993) reported that clocinnamox eliminated μ opioid receptors (labeled by either naltrexone or Tyr-D.Ala-Gly-(Me)Phe-Gly.ol (DAMGO) when administered systemically to mice without changing the affinity of the ligands for this site. This finding suggests that clocinnamox acts as an irreversible opioid receptor ligand.

Mouse Vas Deferens

The vasa deferentia of mice contain each of the three major opioid receptors. When this preparation is stimulated electrically, it releases norepinephrine which, in turn, elicits a twitch of the muscle. Opioid agonists inhibit the release of norepinephrine in the preparation. Both methoclocinnamox and buprenorphine inhibit the electrically elicited twitch of the vas deferens with EC, values of $5x10^{-9}$ M and $2.6x10^{-8}$ M, respectively. Preincubation of the vas deferens with naltrexone will prevent the inhibition induced by either agonist (Aceto et al. 1989; C.B. Smith, unpublished observations). When an inhibitory effect of buprenorphine has been established, its effects are resistant to reversal by repetitive washing of the tissue or addition of competitive antagonists (Schulz and Herz 1976). It is not known if methoclocinnamox shares this property.

Clocinnamox does not act as an agonist in the mouse vas deferens; it prevents fentanyl and alfentanil from acting as opioid agonists. Likewise, clocinnamox insurmountably blocks the effects of U-50,488H, a κ opioid agonist while it only surmountably antagonizes [$Dser^2$,Leu^5,Thr^6]enkephalin (DSLET), a δ receptor agonist (Aceto et al, 1989). Its selectivity in vas deferens is 200- to 300-fold higher for μ agonists (see table 6 of Lewis et al. 1988). It also is a selective antagonist of μ receptor agonists in the guinea pig ileum and rat vas deferens preparations (see table 4 of Lewis et al. 1988).

Metabolism Studies

Incubation of human liver homogenate with methoclocinnamox produced a mixture of clocinnamox, the O-demethylation product, and an N-dealkylated derivative of methoclocinnamox. Thus, metabolism of methoclocinnamox by human liver seems to lie between the cynomolgus monkey (primarily O-demethylation to clocinnamox) and the rat (primarily N-dealkylation). In the cynomolgus monkey, after oral administration of tritiated methoclocinnamox, almost all radioactivity was recovered in the bile in the first 8 hours as polar metabolites, the majority of which (70-80 percent) was the conjugated clocinnamox. When methoclocinnamox was administered subcutaneously, up to 70 percent of methoclocinnamox was found in the unchanged form. This suggests that after oral administration of methoclocinnamox there is a high uptake rate and significant first-pass metabolism.

IN VIVO PREPARATIONS

Rodent Analgesic Studies: Agonist Actions

A variety of opioid agonists show limited effectiveness in different rodent assays of analgesia. A compound may fail to have full effectiveness in a thermal analgesic assay (e.g., tail flick assay) while displaying full effectiveness in an irritant-induced stretching (i.e., writhing) assay. Higher efficacy compounds are active in both assays. Morphine, for example, is fully effective in each assay, although it is most potent in the writhing assay, less potent in the hot plate procedure, and least potent in the tail flick procedure. Both buprenorphine and methoclocinnamox share limited effectiveness in various rodent analgesia assays. Aceto and colleagues (1989) showed that, in the mouse, methoclocinnamox was inactive up to doses of 20-30 mg/kg in both the tail flick and hot plate procedures; it was fully effective at 0.2 mg/kg in the phenylquinone-induced writhing in the mouse. Clocinnamox is inactive in all of these assays. Buprenorphine shows a pattern of agonist effectiveness that is greater than methoclocinnamox but less than morphine (Cowan et al. 1977).

Figure 2(a) shows a dose-effect relationship of methoclocinnamox in the writhing assay; the dose dependence closely approximates that found earlier (Aceto et al. 1989). Figure 2(b) shows the time course of the first fully effective dose (3.2 mg/kg) of methoclocinnamox. It shows

analgesic activity for over 8 hours. In this assay, methoclocinnamox is close to morphine in potency, and it has a much longer duration of action than does buprenorphine (Cowan et al. 1977). It should be noted that methoclocinnamox, due to limited water solubility, can only be given in amounts up to 100 mg/kg systemically. It does not kill mice at this dose.

Methoclocinnamox's analgesic effect in the writhing assay in the mouse is readily *prevented* by some narcotic antagonists. Naltrexone, when given in small doses 15 minutes before methoclocinnamox, reduces the potency of methoclocinnamox considerably (figure 3[a]); 0.01 mg/kg produces a small decrease (4.7-fold) in potency, while 0.1 mg/kg produces a greater decrease in potency (110-fold). Higher doses of methoclocinnamox could not be studied due to its limited solubility, as noted above. The effectiveness of small doses of naltrexone suggests that methoclocinnamox is inducing its analgesic effects through the μ receptor. This supposition was confirmed by administration of 3.2 mg/kg of ß-funaltrexamine (ß-FNA) 24 hours prior to testing (figure 3[b]). Under these dose and pretreatment conditions, ß-FNA antagonizes only μ agonists in this assay. ß-FNA reduces both the potency and the maximum effect of methoclocinnamox to an extent that it is reasonable to assume that virtually all of its analgesic effect in this assay is mediated through the μ receptor. The μ agonist-like effect of methoclocinnamox was confirmed further by the lack of antagonism by either norbinaltorphimine (3.6-fold shift; 32 mg/kg, 24-hour pretreatment), the κ receptor selective competitive antagonist (e.g., Broadbear et al. 1994) or naltrindole (no shift; 1.0-3.2 mg/kg, 30-minute treatment), a δ opioid receptor selective, competitive antagonist. Thus, metho-clocinnamox exerts its analgesic effect in this chemical irritant assay through the μ receptor.

The next issue that was addressed was whether methoclocinnamox's analgesic effect, when established, was readily reversible. Morphine's behavioral effects usually are reversible, while buprenorphine's effects are less so. For example, buprenorphine's discriminative effect is readily prevented but not reversed by naltrexone, while morphine's effect is both prevented and reversed by naltrexone (France et al. 1984). With the mouse writhing assay, a time course of a fully effective dose of morphine, buprenorphine, and methoclocinnamox was established (figure 4). At the time the agonist had its maximal effect, it was combined with increasing doses of naltrexone to establish if the effects could be reversed (figure 5).

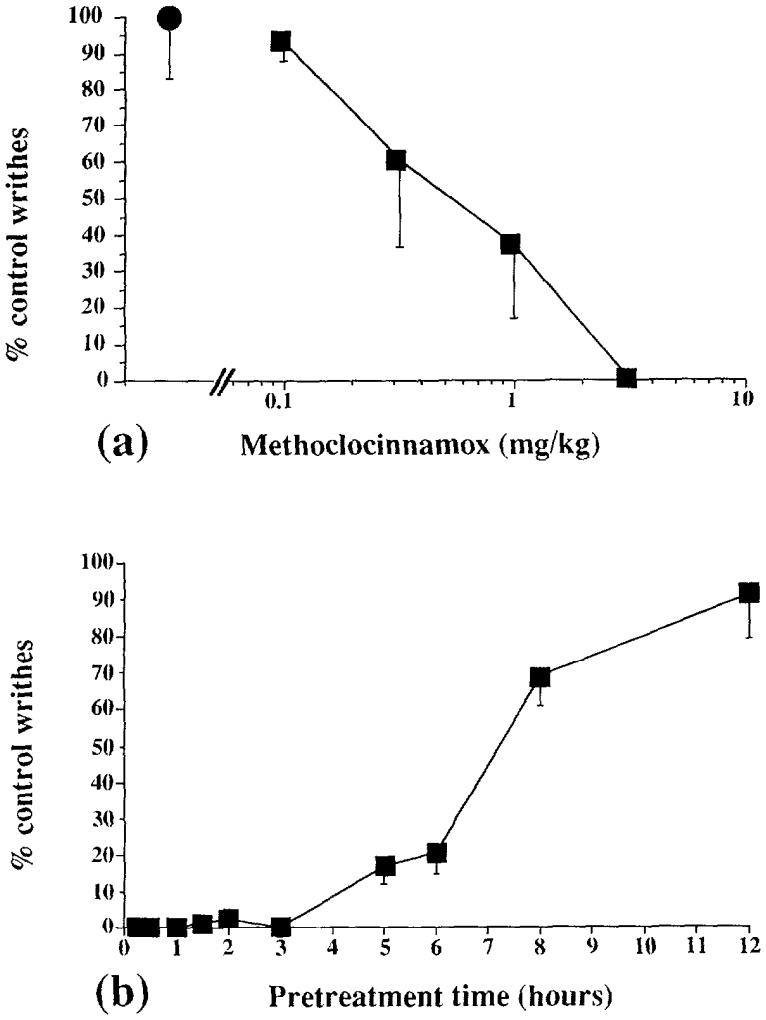

FIGURE 2. *(Panel a): Dose-effect curve of methoclocinnamox following subcutaneous (s.c.) administration and a 15-min pretreatment in the acetic acid-induced writhing assay in the mouse. Sterile water control is shown (●). (Panel b): Time course of the agonist effect of methoclocinnamox in the acetic acid-induced writhing assay in mice. Animals were injected with 3.2 mg/kg methoclocinnamox s.c. and then with 0.4 ml of 0.6 percent acetic acid intraperitoneally 15 min prior to testing. Each data point represents six animals; brackets indicate standard error.*

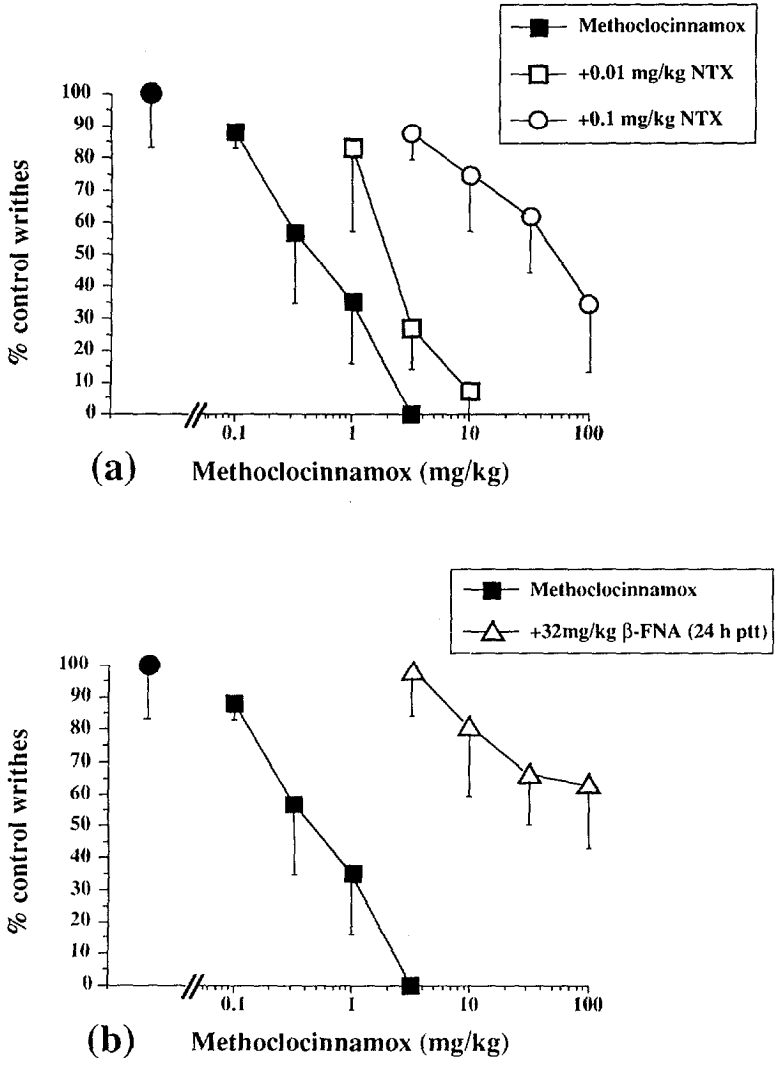

FIGURE 3. *(Panel a): Antagonism of the agonist effect of methoclocinnamox by 0.01(□) and 0.1 (○) mg/kg naltrexone, which was administered 15 min prior to methoclocinnamox. (Panel b): Antagonism of the agonist effect of methoclocinnamox (■) following 24-hour pretreatment with 32 mg/kg ß-FNA(□).. The data in both panels were obtained using the acetic acid-induced writhing assay in mice, and each point represents 6 animals. The sterile water control also is represented(●).*

As shown in figure 5(a), morphine's effects are reversed completely by the same doses of naltrexone that prevents its effects (e.g., Takasuna et al. 1994). As shown in panels (b) and (c), respectively, the analgesic effects of buprenorphine and methoclocinnamox are less readily reversed.

The transition from reversible (i.e., preventable) to irreversible agonist effect can be tracked over the course of action of the three compounds, as shown in figure 4. In figure 4(a), morphine's effect is established rapidly, and it wanes over a 2-hour period. If naltrexone is given 15 minutes before testing, morphine's effects are reversed completely at each time point. Figure 4(b) displays the result of a comparable study of buprenorphine. Buprenorphine has a slower onset and longer duration of analgesic action than does morphine. Naltrexone blocks buprenorphine effectively 30 minutes following buprenorphine administration; at later time points, the antagonism is incomplete. In figure 4(c) are displayed the results with methoclocinnamox. Methoclocinnamox has a more gradual onset of action. Naltrexone reverses methoclocinnamox effectively for approximately 1 hour, and then it is less effective.

These findings show that both buprenorphine and methoclocinnamox go through a process of converting from a reversible agonist action to an antagonist-resistant phase of agonist action. The second phase of effect is not shared with morphine. Thus, their early effects can be prevented readily with the competitive antagonist, naltrexone, and this effect changes with exposure so that the later effects of both buprenorphine and methoclocinnamox are not readily reversed by naltrexone.

Rodent Analgesic Studies: Antagonist Actions

As mentioned above, opioids with limited efficacy may evoke agonist actions in some analgesic assays and be devoid of these actions in others. With some compounds, the agonist action in any assay may wane with time and be displaced by antagonist action. For example, buprenorphine's analgesic action dissipates, leaving a substantial antagonist effect. This allowed Tallarida and Cowan (1982) to use buprenorphine's antagonist action to make the first in vivo calculation of morphine's affinity constant in rats. These relations of efficacy, assay, and temporal changes in effect make for a complex set of opioid actions on behavior.

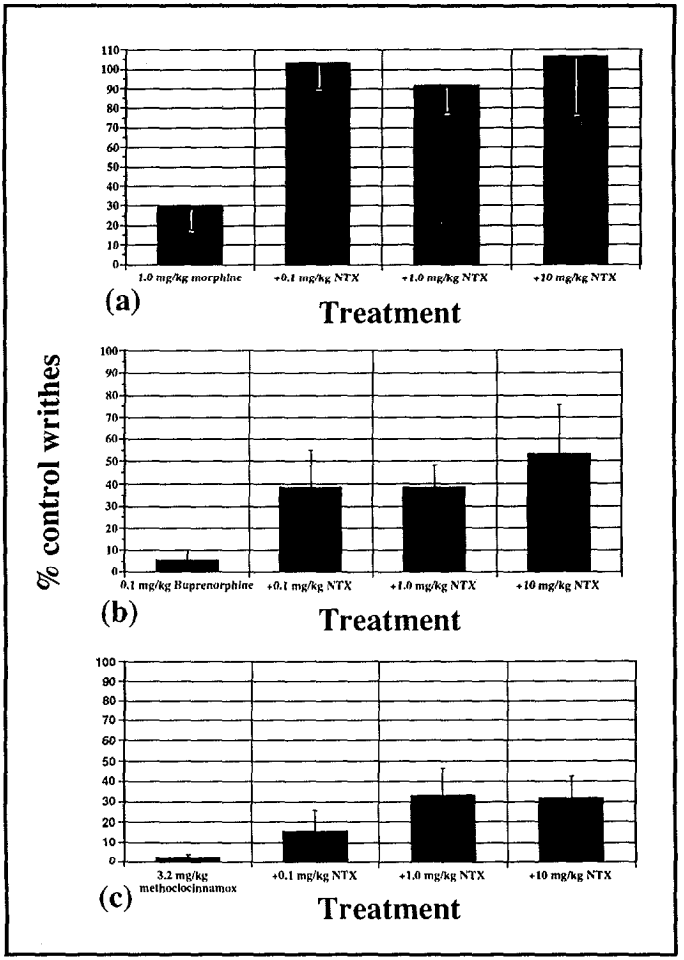

FIGURE 4. *(Panel a): The time course of 1.0 mg/kg morphine (■) in the writhing assay was reversed completely at each time point by the addition of 0.1 mg/kg/ naltrexone (□) 15 min prior to testing. (Panel b): The agonist time course of 0.1 mg/kg buprenorphine (■) is only partially reversed when 0.1 mg/kg naltrexone (Cl) is administered 15 min prior to testing. (Panel c): The agonist time course of 0.32 mg/kg methoclocinnamox (■) is only partially reversed by the administration of 0.1 mg/kg naltrexone (□).*

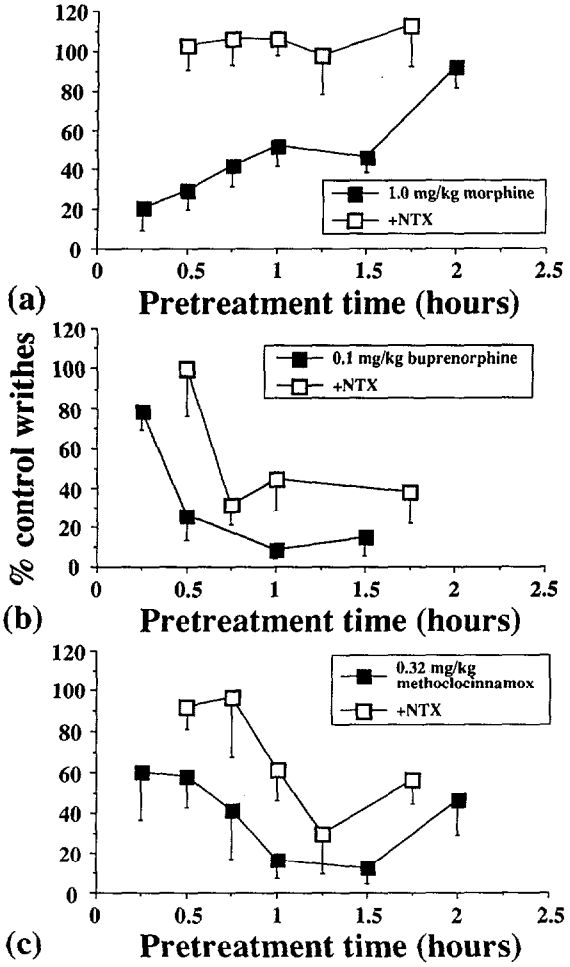

FIGURE 5. *(Panel a): Reversibility of the peak agonist effect obtained with morphine was demonstrated by administering 0.1, 1.0, and 10 mg/kg naltrexone after a 30-min pretreatment with 1.0 mg/kg morphine and testing 15 min later. Each bar represents the results of six mice in the writhing assay. (Panel b): Naltrexone had only partial and nondose-related success in reversing the peak agonist effect of buprenorphine when it was administered 90 min after 0.1 mg/kg buprenorphine was given. (Panel c): As with buprenorphine, naltrexone produced a small and nondose-related reversal of methoclocinnamox's peak agonist effect when administered 2 hours after 3.2 mg/kg methoclocinnamox.*

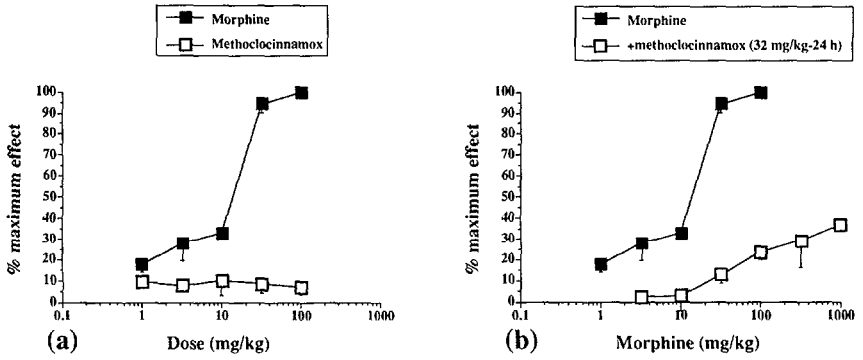

FIGURE 6. *(Panel a): The effects of morphine (■) and methoclocinnamox (□) following cumulative dosing in the warm water (50 °C) tail withdrawal procedure in mice. Panel b: Twenty-four hours after administration, 32 mg/kg methoclocinnamox (□) insurmountably antagonized the agonist effect of morphine (■) up to lethal doses in the warm water tail withdrawal assay.*

Methoclocinnamox is without agonist effect when given in behaviorally active dose ranges, in a 50 °C tail withdrawal assay in the mouse (Aceto et al. 1989) (see figure 6[a]). When administered as a pretreatment to morphine, it acts as an insurmountable antagonist (see figure 6[b]). Clocinnamox also is an insurmountable antagonist, appearing to be more potent as a morphine antagonist than methoclocinnamox (Aceto et al. 1989; E. Butelman, unpublished observations; Comer et al. 1992).

Methoclocinnamox's antagonist action at high doses is long lasting in the mouse. A dose of 32 mg/kg is capable of producing a substantial antagonism of morphine even 4 days after its administration (figure 7). Clocinnamox also has a long duration of action following a single systemic dose (Aceto et al. 1989; Comer et al. 1992). As noted above, some of methoclocinnamox's antagonist activity may be due to its metabolic conversion to clocinnamox.

The spectrum of methoclocinnamox's opioid antagonist action has been examined in the mouse. Delta and κ receptor-mediated analgesia may be detected in the chemical-irritant writhing assay. Twenty-four hours following a 32 mg/kg dose of methoclocinnamox, its agonist effect has dissipated but, as shown in the tail withdrawal assay, it remains active as an antagonist (figure 7). It antagonizes both morphine and etonitazene under these conditions, producing 3- to 10-fold reductions in potency of

these agonists (figure 8[a] and 8[b]). Under these same conditions, it is ineffective against U69,593 or bremazocine (κ agonists) and against the systemically active δ opioid BW 373U86 (figures 8[c]-8[e]). Thus, in the mouse, it appears that methoclocinnamox exerts both agonist and antagonist actions selectively through the μ receptor. This may be a circumstance where methoclocinnamox and buprenorphine differ; buprenorphine is a very potent κ antagonist in a variety of animal preparations (e.g., Dykstra and Negus, in press; Negus and Dykstra 1988). This difference needs to be explored in a variety of behavioral circumstances that directly compare buprenorphine and methoclocinnamox.

STUDIES IN RHESUS MONKEYS

Analgesia: Agonist Followed by Antagonist Action

A variety of opioids have been assessed in a thermal antinociception assay in rhesus monkeys (e.g., Dykstra and Woods 1986; Woods et al. 1992). In this assay, a latency measure of tail withdrawal to 40 °C (control), 50 °C, or 55 °C water indicates analgesic activity. Strong μ (Walker et al. 1993) or κ agonists (Dykstra et al. 1987; France et al. 1994) produce maximum effects with both 50 °C and 55 °C assessments.

Alpha-2 agonists (Butelman and Woods 1993) and NMDA-type excitatory amino acid antagonists (France et al. 1989, 1990) also produce this pattern of activity. Opioids with apparent reduced efficacy produce an analgesic effect at the lower temperature of water without an accompanying effect at 55 °C (Walker et al. 1993). Based on the data presented in **the Rodent Analgesic Studies: Agonist Actions** section, it might be anticipated that methoclocinnamox would produce effects of this kind and, indeed, this pattern of result is obtained (figure 9). Methoclocinnamox's analgesic effects increase over the first 2 hours at 50 °C, and maximum effects continue for another 1.5 hours before diminishing to control at the fifth hour. During this time, there is no analgesic effect demonstrated at 55 °C; the effect, consequently, is not the result of a loss of capacity to respond to thermal stimuli.

Methoclocinnamox's antagonist effect was studied by examining its effects in combination with alfentanil, a short-acting μ agonist, at 55 °C. A single dose of 1 mg/kg methoclocinnamox was administered, and its antagonist effect was examined over the course of a week and a half

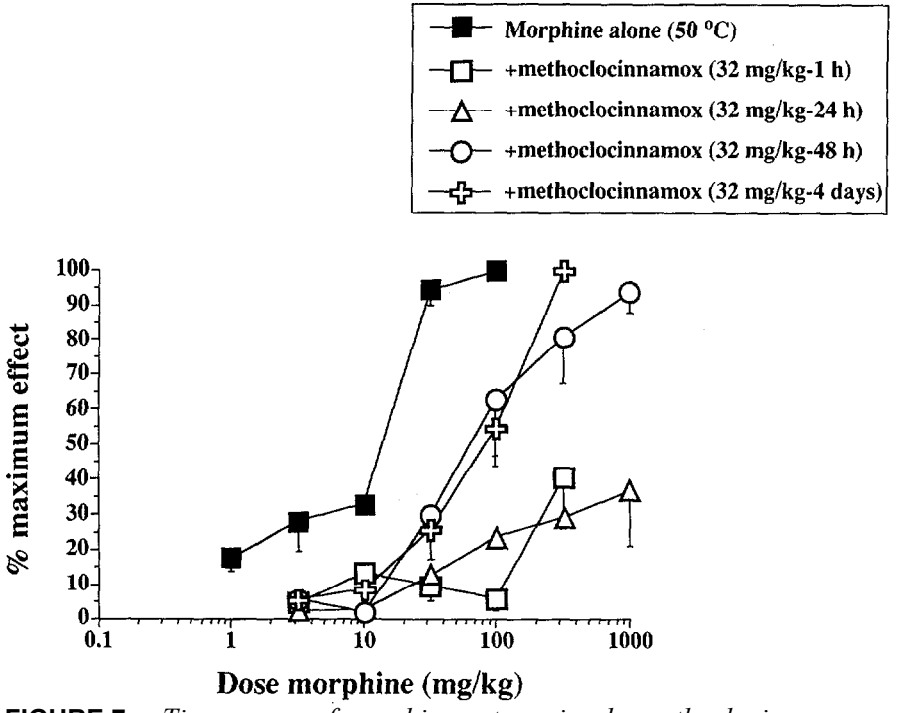

FIGURE 7. *Time course of morphine antagonism by methoclocinnamox when morphine was given alone (■) and following a 1-hour (□), 24-hour (△), or 48-hour (○), and 4-day (✣) pretreatment with methoclocinnamox in the warm water (50 °C) tail withdrawal procedure in mice*

(figure 10). Methoclocinnamox's antagonist effect is demonstrable at 24 hours, when it produces a 10-fold reduction in alfentanil's analgesic potency. At 48 hours, its effects are even larger, and a full effect of alfentanil cannot be obtained, even with 10 mg/kg of alfentanil. Under control conditions, alfentanil produces severe respiratory depression at doses of 0.32 mg/kg. Thus, signifant protection against lethal doses of alfentanil is produced for nearly a week with a single systemic dose of methoclocinnamox. Eleven days following its administration, normal sensitivity to alfentanil's analgesic effects has returned.

Respiration: Limited Depressant Effects

Among the major opioid receptor types, μ agonists are the only set of compounds that affect respiratory control in primates (Butelman et al. 1993; France and Woods 1990; Howell et al. 1988). Strong agonists of

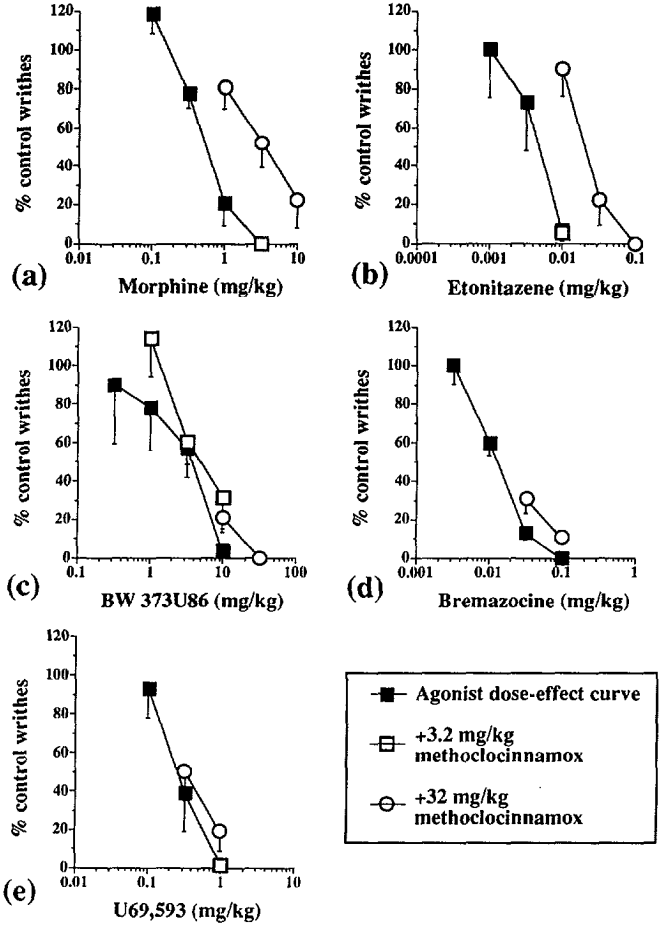

FIGURE 8. *(Panel a): Morphine and etonitazene's dose-effect curves (■) following a 24-hour pretreatment with 3.2 (□-not shifted) and 32 mg/kg methoclocinnamox (○-shifted~3-fold) in the acetic acid-induced writhing assay in mice. (Panel b): The dose-effect curves of BW 373 U86, bremazocine, and U69,593 (■) were not affected by 24-hour-pretreatment with 32* mg/kg *methoclocinnamox (□).*

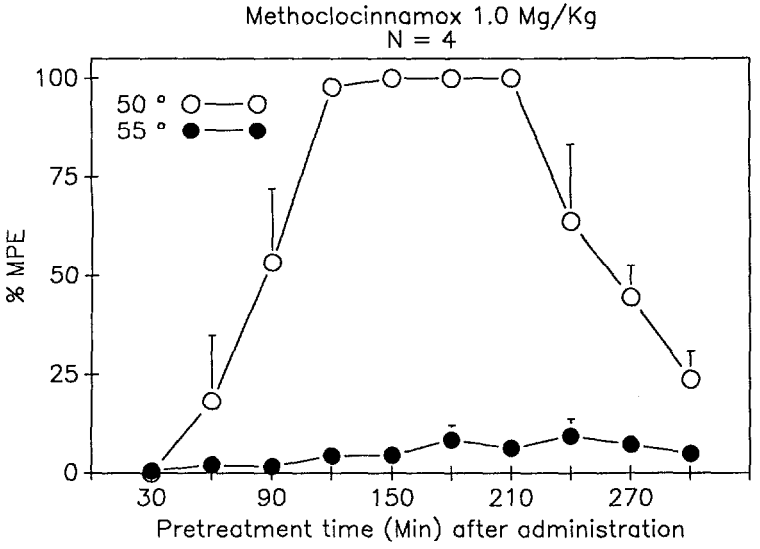

FIGURE 9. *Time course for the antinociceptive effects of methoclocinnamox (1.0 mg/kg) in the warm water (50 °C and 55 °C) tail withdrawal assay in rhesus monkeys*

the μ type are capable of producing apnea. There are only a few preliminary observations with methoclocinnamox. An intermediate dose (0.32 mg/kg) of methoclocinnamox produces a modest reduction in CO_2-stimulated respiration; the reduction lasts for over 10 hours. If a competitive opioid antagonist, quadazocine, is co-administered with methoclocinnamox, the respiratory depression is attenuated, suggesting that this effect is μ opioid receptor mediated. Studies of the antagonist effects of methoclocinnamox over its entire time course are in order and will be very interesting. It nevertheless is clear that methoclocinnamox will exert no serious respiratory depressant effects and may protect significantly against opioid overdose for long periods of time following the administration of a single dose. Buprenorphine also has a limited respiratory depressant effect in rhesus monkeys (Woods et al. 1992). Its antagonist effects against other opioids apparently have not been studied.

Discriminative Stimulus Effects: Mu Agonist-Like Actions

Opioids can induce a variety of discriminative effects that depend upon the training conditions of the subject (e.g., Comer et al. 1991). In monkeys trained to discriminate codeine from vehicle,

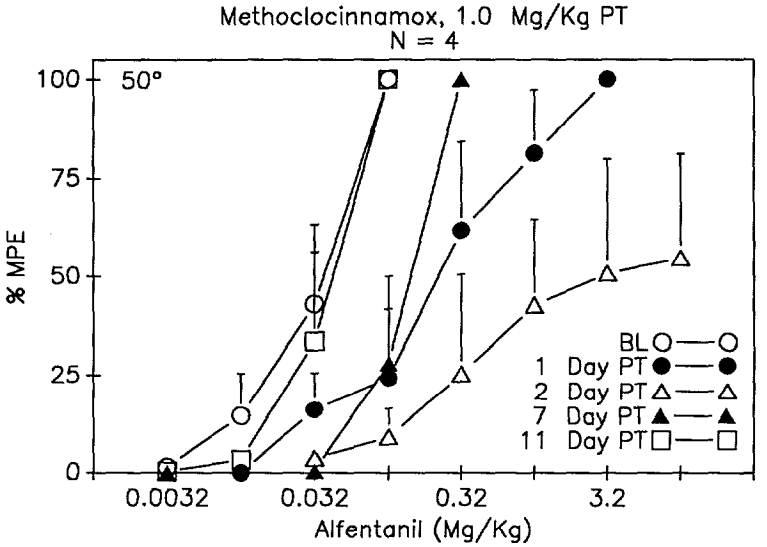

FIGURE 10. *Time course for the antagonist effcts of methoclocinnamox (1.0 mg/kg) on alfentanil-induced untinociception in the warm water (50 °C) tail withdrawal assay in rhesus monkeys. Alfentunil dose-effect curves were determined before methoclocinnamox (baseline = BL), and 1, 2, 7, and 11 days after pretreatment.*

methoclocinnamox produced an incomplete substitution for the training drug (Aceto et al. 1989). Buprenorphine has discriminative effects similar to other μ agonists (Young et al. 1984). Clocinnamox fails to substitute for codeine in monkeys (Aceto et al. 1989).

Reinforcing Effects and Pretreatment Effects on Alfentanil- and Cocaine-Reinforced Responding

Opioids that are selective agonists at the μ receptor can serve as reinforcers; agonists at the κ and δ receptor do not (Woods et al. 1993). Opioids with limited efficacy at the μ receptor tend to show more variability in their capacity to be effective reinforcers across assessment conditions (Woods et al. 1993). When methoclocinnamox was evaluated initially under conditions in which a variety of μ agonists served as reinforcers, it maintained responding only at rates slightly above those maintained by saline (Aceto et al. 1989). Using a procedure that requires less capacity of the reinforcer (Winger et al. 1989), methoclocinnamox is

more effective as a reinforcer (figure 11). In this study, methoclocinnamox is almost as effective as alfentanil but was slightly less potent on a weight basis. Buprenorphine has a very similar pattern of effects across the two procedures (Winger et al. 1992).

When buprenorphine is administered prior to the opportunity to self-inject alfentanil, it suppresses the self-injection response. It is remarkably selective in suppressing opioid-reinforced responding, relative to cocaine-reinforced responding (Winger et al. 1992). Preliminary experiments with methoclocinnamox suggest that it, too, has this pattern in rhesus monkeys (figure 12). In this study, a single dose of methoclocinnamox was administered prior to a period of drug self-injection wherein a set of alfentanil doses was assessed for reinforcing effect. Shortly therafter (30 minutes), the reinforcing potency of alfentanil was reduced by approximately 30-fold. Over the next 5 days, the potency of alfentanil returned to control conditons. Thus, a considerable protection against alfentanil's reinforcing effects was afforded by a single dose of methoclocinnamox. The reinforcing effect of cocaine was not altered by this dose of methoclocinnamox (figure 13), although a slightly higher dose of methoclocinnamox reduced cocaine self-injection as well. The suppression of cocaine-reinforced responding was not of long duration. It should be noted that the doses of methoclocinnamox and buprenorphine that are active in these self-administration procedures are within the range of doses that are effective in altering other behaviors in rhesus monkeys. Clocinnamox also selectively changes alfentanil-reinforced responding relative to cocaine-reinforced responding (Winger, unpublished observations).

Observations in Morphine-Dependent Rhesus Monkeys

In morphine-dependent rhesus monkeys given morphine, 3 mg/kg/4 hours, a withdrawal syndrome is reliably detected if a single injection is omitted. Under these conditions, methoclocinnamox suppresses nearly all signs of opioid withdrawal by a dose of 0.8 mg/kg. The suppression of withdrawal is dose dependent over a range of 0.05-0.8 mg/kg (see figure 2 of Aceto et al. 1989). If methoclocinnamox (0.8 mg/kg) is substituted for a 3 mg/kg morphine injection, withdrawal signs emerge slowly over a 3-day period. These withdrawal signs are suppressed incompletely by morphine (Aceto, personal communication, 1989).

Clocinnamox has no capacity to suppress withdrawal. It exacerbates withdrawal, and morphine administration is ineffective in reversing the

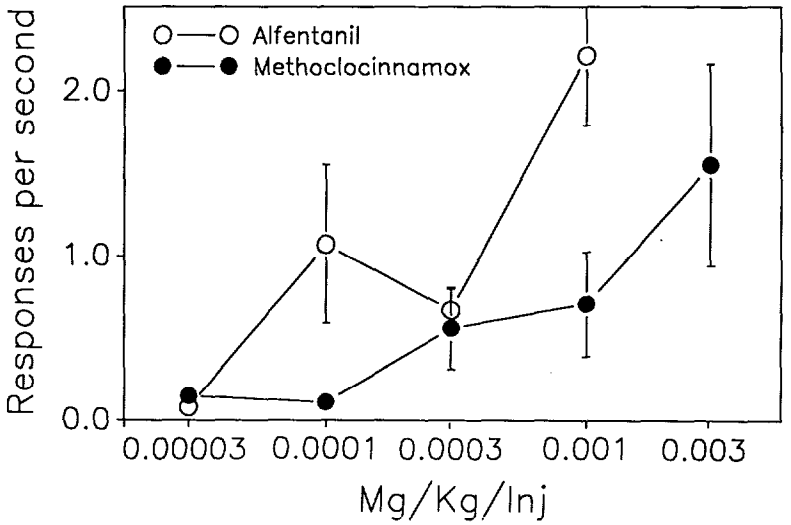

FIGURE 11. *Rates of responding maintained by intravenous alfentanil (○) or by methoclocinnamox (●). Data are the mean and standard error of the mean (SEM) (vertical bars) of four monkeys that had either alfentanil or methoclocinnamox available as a consequence of responding on an available lever. The schedule of drug delivery was amd-ratio 30 timeout 45 sec in a paradigm in which four doses* of *either drug were available, each for 20 injections or 25 min in a 130-min session. A 10-min blackout period separated each component of these sessions. Doses were altered by changing the duration of the infusion within a single session. A larger dose range (five doses of methoclocinnamox were studied) was evaluated by increasing the concentration of the drug in a given session. The baseline drug was alfentanil, and the data shown for alfentanil are the mean of the sessions that immediately preceeded single session substitutions of methoclocinnamox.*

NOTE: Abscissa is the rate of responding during the time a red light signalled drug availability in responses/sec. Ordinate is the dose of alfentanil or methoclocinnamox that was delivered with each injection.

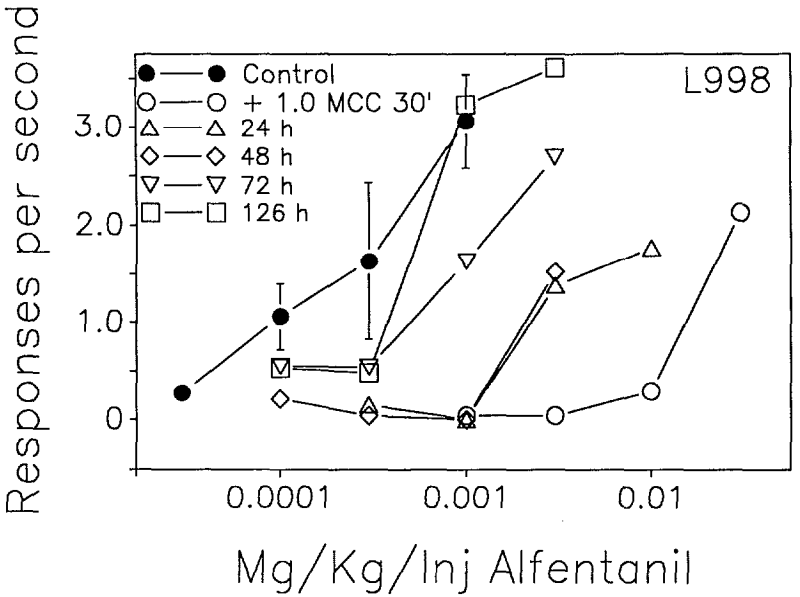

FIGURE 12. *Effects of 1.0 mg/kg methoclocinnamox on rates of responding maintained by alfentanil in monkey L998. Control rates of alfentanil-maintained responding (●) are the mean and SEM of three sessions that preceeded administration of methoclocinnamox. The pretreatment was given s.c. 30 min prior to the initiation of a session of alfentanil availability. Observations were made of the effect of the administration of methoclocinnamox on alfentanil-maintained responding over a period of 126 hours. The details of the schedule are given in figure 11. Four doses of alfentanil were available in each session, each dose for 20 injections or 25 min. A 10-min blackout occurred between availability of each dose. Doses within a session were varied by altering the infusion duration. Larger dose ranges were examined across sessions by increasing the concentration of alfentanil available. Two 130-min sessions were scheduled each day, one at 10:00 am and one at 4:00 pm.*

NOTE: Abscissa is the rate of responding in responses per set during the time a red light signalled alfentanil availability. Ordinate is the dose of alfentanil that was delivered with each injection.

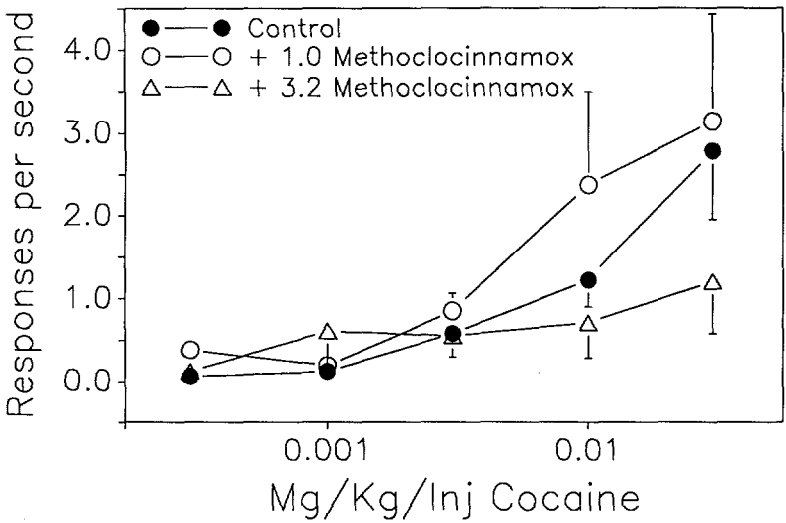

FIGURE 13. *Rates of responding maintained by cocaine under conditions* in *which no pretreatment was given(●); 1.0 mg/kg methoclocinnamox was administered 30 min prior to the initiation of a session of cocaine availability (○), or 3.2 mg/kg methoclocinnamox was administered 30 min prior to the initiation of such a session (△). The data points are the mean and SEM of four monkeys, three of whom received methoclocinnamox and one who received the pretreatment intravenously. One of the monkeys who received s.c. pretreatments was studied using a dose range of 0.0003 to 0.01 mg/kg/inj cocaine; the other three received a dose range of 0.001 to 0.3 mg/kg/inj. Cocaine availability and the schedule parameters are as described for figure 11. The cocaine data are from sessions that immediately preceeded those in which methoclocinnamox was administered as a pretreatment.*

NOTE: Abscissa is the rate of responding in responses per set during the time a red light signalled cocaine availability. Ordinate is the dose of cocaine that was delivered with each injection.

withdrawal exacerbated by clocinnamox. Buprenorphine is similar to clocinnamox in both failing to reverse morphine abstinence and preventing its reversal with morphine (Woods and Gmerek 1985).

It has been difficult to demonstrate opioid dependence on buprenorphine in rhesus monkeys (e.g., Gmerek and Woods 1985; Woods and Gmerek 1985). Studies of direct methoclocinnamox dependence have not been carried out. It may be difficult to demonstrate dependence under conditions of chronic administration if a significant portion of methoclocinnamox is metabolized to clocinnamox. These studies nevertheless are extremely important, and much will depend upon the regimen of chronic administration.

DISCUSSION

Methoclocinnamox has a μ partial agonist profile of activity in mice and rhesus monkeys. Its agonist effects appear, at least under some circumstances, to be resistant to antagonism by competitive narcotic antagonists. This property has been studied most thoroughly in mouse analgesic preparations. It appears to have greater agonist effectiveness than buprenorphine in rhesus monkeys, is more effective as an analgesic, and suppresses morphine abstinence (at least initially). The agonist effect is not sufficient, however, to produce significant respiratory toxicity. Rather, during its course of action, methoclocinnamox will protect against the toxicity produced by strong μ agonists. When viewed as an antagonist, methoclocinnamox can provide significant protection against opioid agonists over a number of days when the analgesic, respiratory, or reinforcing effects of opioids are evaluated.

There are a variety of questions that need to be investigated in the future. There have been no studies of chronic toxicity carried out with methoclocinnamox. Indeed, its conversion to clocinnamox and its acute actions suggest that its chronic effects are complex. These issues will need to be addressed thoroughly if researchers are to understand the capacity of methoclocinnamox to deter opioid abuse.

Nevertheless, in the authors' opinion, methoclocinnamox deserves consideration as a novel therapeutic approach to heroin abuse. Its limited efficacy at μ receptors suggests that, if given appropriately, compliance will be greater; perhaps it will be more effective than buprenorphine. It should have little if any toxicity, as surmised from its demonstrated

opioid partial agonist profile and its limited efficacy and solubility. Finally, based on its long duration of action in rhesus monkeys, it might require less frequent administration than either methadone or buprenorphine.

REFERENCES

Aceto, M.D.; Bowman, E.R.; May, E.L.; Harris, Ls.; Woods, J.H.; Smith, C.B.; Medizhradsky, F.; and Jacobson, A.E. Very long-acting narcotic antagonists: The 14ß-p-substituted cinnamoylaminomorphinones and their partial mu agonist codeinone relatives. *Arz Forsch Drug Res* 39(5):570-575, 1989.

Broadbear, J.H.; Negus, S.S.; Butelman, E.R.; de Costa, B.R.; and Woods, J.H. Differential effects of systemically administered nor-binaltorphimine (nor-BNI) on κ-opioid agonists in the mouse writhing assay. *Psychopharmacology* 115:311-319, 1994.

Burke, T.F.; Medzihradsky, F.; Woods, J.H.; and Lewis, J.W. Behavioral and biochemical characterization of irreversible opioid receptor antagonists. *FASEB J* 7(3):A487, 1993.

Butelman, E.R., and Woods, J.H. Effects of clonidine, dexmedetomidine and xylazine on thermal antinociception in rhesus monkeys. *J Phamacol Exp Ther* 264(2):762-769, 1993.

Butelman, E.R.; France, C.P.; and Woods, J.H. Apparant pA_2 analysis on the respiratory depressant effects of alfentanil, etonitazene, ethylketocyclazocine (EKC) and Mr2033 in rhesus monkeys. *J Pharmacol Exp Ther* 264(1):145-151, 1993.

Comer, S.D.; Burke, T.F.; Lewis, J.W.; and Woods, J.H. Clocinnamox: A novel, systemically-active, irreversible opioid antagonist. *J Pharmacol Exp Ther* 262(3):1051-1056, 1992.

Comer, S.D.; France, C.P.; and Woods, J.H. Training dose: Influence in opioid drug discrimination. In: Harris, L.S., ed. *Drug Discrimination: Applications to Drug Abuse Research.* National Institute on Drug Abuse Research Monograph 116. DHHS Pub. No. (ADM)92-1878. Washington, DC: Supt. of Docs., U.S. Govt. Print. Off., 1991. pp. 145-161.

Cowan, A.; Lewis, J.W.; amd Macfarlane, I.R. Agonist and antagonist properties of buprenorphine, a new antinociceptive agent. *Br J Pharrnacol* 60:537-545, 1977.

Dykstra, L.A.; Gmerek, D.E.; Winger, G.; and Woods, J.H. Kappa opioids in rhesus monkeys: I. Diuresis, sedation, analgesia and discriminative stimulus effects. *J Pharmacol Exp Ther* 242:413-420, 1987.

Dykstra, L.A., and Woods, J.H. A tail withdrawal procedure for assessing analgesic activity in rhesus monkeys. *J Pharmacol Meth* 15:263-269, 1986.

Dykstra, L.A., and Negus, S.S. Behavioral and pharmacological determinants of buprenorphine. In: Cowan, A., and Lewis, J., eds. *Buprenorphine.* New York: John Wiley and Sons, in press.

France, C.P.; Jacobsen, A.E.; and Woods, J.H. Discriminative stimulus effects of reversible and irreversible opiate agonists: Morphine, oxymorphazone and buprenorphine. *J Pharmacol Exp Ther* 230(3):652-657, 1984.

France, C.P.; Medzihradsky, F.; and Woods, J.H. Comparison of κ opioids in rhesus monkeys: Behavioral effects and receptor binding affinities. *J Pharmacol Exp Ther* 268:47-58, 1994.

France, C.P.; Snyder, A.M.; and Woods, J.H. Analgesic effects of phencyclidine-like drugs in rhesus monkeys. *J Pharmacol Exp Ther* 250(1):197-201, 1989.

France, C.P., and Woods, J.H. Respiratory effects of receptor-selective opioids in rhesus monkeys. In: Quirion, R.; Jhamandas, K.; and Gianoulakis, C., eds. *The International Narcotics Research Conference (INRC) '89.* New York: A.R. Liss, 1990. pp. 295-298.

Gmerek, D.E., and Woods, J.H. Effects of ß-funaltrexamine in normal and morphine-dependent rhesus monkeys: Observational studies. *J Pharmacol Exp Ther* 235:296-301, 1985.

Howell, L.L.; Bergman, J.; and Morse, W.H. Effects of levorphanol and several κ-selective opioids on respiration and behavior in rhesus monkeys. *J Pharmacol Exp Ther* 245(1):364-372, 1988.

Lewis, J.; Smith, C.; McCarthy, P.; Walter, D.; Kobylecki, R.; Myers, M.; Haynes, A.; Lewis, C.; and Waltham, K. New 14-amino-morphinones and codeinones. In: Harris, L.S., ed. *Problems of Drug Dependence 1988.* National Institute on Drug Abuse Research Monograph 90. DHHS Pub No. (ADM)89-1605. Washington, DC: Supt. of Docs., U.S. Govt. Print. Off., 1988. pp. 136-143.

Negus, S.S., and Dykstra, L.A.κ antagonist properties of buprenorphine in the shock titration procedure. *Eur J Pharmacol* 156:77-86, 1988.

Schulz, R., and Herz, A. The guinea pig ileum as an *in vivo* model to analyze dependence liability of narcotic drugs. In: Kosterlitz, H.W., ed. *Opiates and Endogenous Opioid Peptides.* Amsterdam: Elsevier, 1976. pp. 319-326.

Takasuna, M.; Negus, S.S.; and Woods, J.H. Opioid pharmacology of the antinociceptive effects of loperamide in mice. *Behav Pharmacol* 5:189-195, 1994.

Tallarida, R.J., and Cowan, A. The affinity of morphine for its pharmacologic receptor *in vivo*. *J Pharmacol Exp Ther* 222(1):198-201, 1982.

Walker, E.A.; Butelman, E.R.; DeCosta, B.R.; and Woods, J.H. Opioid thermal antinociception in rhesus monkeys: Receptor mechanisms and temperature dependency. *J Pharmacol Exp Ther* 267:280-286, 1993.

Winger, G.; Palmer, R.K.; and Woods, J.H. Drug-reinforced responding: Rapid determination of dose-response functions. *Drug Alcohol Depend* 24:135-142, 1989.

Winger, G., Skjoldager, P., and Woods, J.H. Effects of buprenorphine and other opioid agonists and antagonists on alfentanil- and cocaine-reinforced responding in rhesus monkeys. *J Pharmacol Exp Ther* 261(1):311-317, 1992.

Woods, J.H.; Winger, G.; and France, C.P. Use of *in vivo* apparent pA_2 analysis in assessment of opioid abuse liability. *Trends Pharmacol Sci* 13:282-285, 1992.

Woods, J.H.; France, C.P.; Winger, G.; Bertalmio, A.J.; and Schwarz-Stevens, K. Opioid abuse liability assessment in rhesus monkeys. In: Herz, A.; Akil, H.; and Simon, E.J., eds. *Opioids II.* Berlin: Springer-Verlag, 1993. pp. 449-469.

Woods, J.H., and Gmerek, D.E. Substitution and primary dependence studies in animals. *Drug Alcohol Depend* 14:233-247, 1985.

Young, A.M.; Stephens, K.R.; Hein, D.W.; and Woods, J.H. Reinforcing and discriminative stimulus properties of mixed agonist-antagonist opioids. *J Pharmacol Exp Ther* 229:118-126, 1984.

ACKNOWLEDGMENTS

This work was supported by USPHS grant no. DA-00254.

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ACEA-1011, a Novel NMDA Receptor/Glycine Site Antagonist, Produces Antinociception but Not Tolerance in the Formalin Test in Mice

Kabirulluh Lutfy, John F. W. Keana, and Eckard Weber

INTRODUCTION

Formalin-induced tonic nociception, which may resemble human postoperative pain, is used widely as a method for evaluating the antinociceptive effect of a mild analgesic (Hunskaar and Hole 1987; Hunskaar et al. 1985). Microinjections of diluted formalin into one of the hind paws in mice produce a biphasic nociceptive response (Rosland et al. 1990; Shibata et al. 1989; Vaccarino et al. 1993) consisting of a transient early phase followed by a tonic late phase, suggesting different pain pathways (Dubuisson and Dennis 1977; Hunskaar and Hole 1987; Hunskaar et al. 1985; Shibata et al. 1989). For example, it is known that morphine and other centrally acting analgesics produce antinociception in both phases of the formalin test, whereas most of the steroidal and nonsteroidal anti-inflammatory drugs are active only in the late phase of the test (Hunskaar and Hole 1987; Hunskaar et al. 1985; Shibata et al. 1989). Tolerance to the analgesic effect of morphine has been demonstrated following acute and chronic administration of the drug (Huidoboro et al, 1976; Kometsky and Bain 1968; Lutfy and Yobum 1991; Way et al. 1969). There are few articles (Abbot et al. 1981, 1982), however, discussing the development of tolerance in the formalin test, which has more clinical relevance than the acute procedures such as tail flick or hot plate tests.

According to the results of recent studies, *N*-methyl-D-Aspartate (NMDA) receptors may play a role in the modulation of tonic pain (Coderre and Melzack 1992; Elliott et al. 1991; Millan and Seguin 1993; Murray et al. 1991; Nasstrom et al. 1992; Vaccarino et al. 1993; Yamamoto and Yaksh 1992). For example, the co-existence of glutamate with substance P in dorsal root ganglion neurons supports this opinion (Battaglia and Rustioni 1988). Furthermore, it has been demonstrated

that subcutaneous (s.c.) injections of formalin produce an immediate increase in the extracellular level of glutamate and aspartate in the dorsal lumbar spinal cord of freely moving rats (Skilling et al. 1988). In addition, it has been reported that a microinjection of formalin into the hind paw or repeated electrical stimulation of C-fibers in the rat causes a progressive increase in firing rate (i.e., wind-up) of the dorsal horn neurons that is blocked by the competitive and noncompetitive NMDA receptor antagonists (Dickenson and Sullivan 1990; Haley et al. 1990; Woolf and Thompson 1991). Therefore, it is expected that the NMDA receptor antagonists show analgesic activities in the formalin-induced tonic pain; however, the phencyclidine-like side effects common to most NMDA receptor antagonists impede the effective therapeutic use of these compounds.

It is well known that activation of the glycine modulatory site coupled to the NMDA receptor is required for the activation of NMDA receptors (Johnson and Ascher 1987; Kleckner and Dingledine 1988; Mayer et al. 1989; Thomson et al. 1989). It has been shown that 7-chloro-kynurenate, an antagonist for the glycine modulatory site associated with the NMDA receptor, blocked the wind-up phenomenon (Dickenson and Ayder 1991). Thus, another approach to produce analgesia would be to antagonize the glycine modulatory site on the NMDA receptor. Recently, an initial pharmacological characterization of 5-chloro-7-trifluoromethyl-1,2,3,4-tetrahydroquinoxaline-2,3-dione (ACEA-1011), a novel competitive antagonist of the NMDA receptor/glycine site, has been reported (Lufty et al. 1994). The present study used ACEA- 1 to test the hypothesis that inhibition at the NMDA receptor/glycine site might be a viable means of producing antinociception without tolerance. For comparison, in parallel studies, the analgesic and tolerance-inducing effects of morphine were examined in the formalin-induced tonic pain model in Swiss Webster mice.

MATERIALS AND METHODS

Subjects

Male Swiss Webster mice weighing 25-35 grams obtained from Simonsen Laboratories, Inc. (Gilroy, CA) were used in all experiments. Four to six mice were maintained in a cage with free access to food and water under a 12-hour light/12-hour dark cycle. Mice were housed for at least 5 days prior to experimentation and used only once. All

experiments were conducted during the light cycle in a blind manner, in which the observers were unaware of different treatments.

Formalin Test

A modification of a previously described method (Hunskaar et al. 1985) was used. Briefly, mice were placed in Plexiglas jars for at least 1 hour for accommodation to the experimental condition. Formalin (20 µl of a 5-percent formaldehyde solution in saline) was injected into the dorsal surface of the right hind paw using a microsyringe with a 27-gauge needle. Mice then were transferred to the Plexiglas jars and immediately observed for licking or biting of the injected paw for 1 hour. The amount of time that the mouse spent licking or biting the injected paw was recorded for every 5-minute period. Saline-injected (20 µl) and untreated control mice also were included in this study in order to examine the effect of injection-induced or spontaneous licking behavior of the mice.

Effect of ACEA-1011 and Morphine in the Formalin Test

Following the accommodation period, mice were weighted and injected either with ACEA-1011 (1.0-30.0 mg/kg, intraperitoneally [i.p.]; N = 12-15 mice/dose) or morphine (1.5-4.0 mg/kg, s.c.; N = 5-11 mice/dose). Control mice were injected with either dimethylsulfoxide (DMSO) (1 ml/kg, i.p.) or saline (10 ml/kg, s.c.), respectively. Thirty minutes later, mice were injected with formalin, transferred to the Plexiglas jars, and immediately observed for licking or biting of the injected paw for 1 hour.

Tolerance to ACEA-1011 and Morphine Analgesia in the Formalin Test

Mice were injected daily for a period of 6 days with either ACEA-1011 (20 mg/kg, i.p.; N = 10 mice) or morphine (20 mg/kg, s.c.; N = 13 mice). Control mice were injected with either DMSO (1 ml/kg, i.p.; N = 12 mice) or saline (10 ml/kg, s.c.; N = 13 mice), respectively. In order to measure the licking or biting behaviors following a formalin injection in naive mice, one group of mice (N = 5) was left untreated; On day 7, mice from ACEA-1011 - and DMSO-treated groups were injected with ACEA-1011 (20 mg/kg, i.p.), while mice from morphine- and saline-treated groups received morphine (3 mg/kg, s.c.). The untreated group, once again, did not receive any of the treatments. Thirty minutes later, mice of all the groups (including the untreated mice) were injected with formalin

into the dorsal surface of the right hind paw, transferred to the Plexiglas jars, and immediately observed for licking or biting of the injected paw for 1 hour.

Drugs

Morphine sulfate was purchased from Research Biochemicals Inc. (Natick, MA). ACEA-1011 was prepared by Dr. John F.W. Keana at the University of Oregon. Morphine and ACEA-1011 were dissolved in saline (0.9 percent NaCl) and DMSO, respectively. Wherever DMSO was used as the vehicle, the solutions were made in such a way that the injection volume was 1 µl/g of body weight per mouse. Morphine doses are expressed as the freebase.

Data Analysis

The mean time spent licking or biting during the early (0-5 minutes) and late (15-50 minutes) phases of the formalin test was expressed as percent of control. The vehicle-treated groups, saline- or DMSO-treated mice, were considered controls. The data shown for the late phase are the average of seven 5-minute periods (i.e., 15-50 minutes following formalin injections). Linear regression analysis was used to calculate the ED, and 95 percent confidence limits (CLs) by the method of Tallarida and Murray (1986).

One-way analysis of variance (ANOVA) was used, followed by post hoc Newman-Keuls multiple comparisons to reveal significant effects of the groups.

RESULTS

Formalin injections into the dorsal surface of the right hind paw of Swiss Webster mice produced a biphasic nociceptive response (i.e., vigorous licking or biting of the injected paw) (figure 1). There were two statistically significant periods of high licking, an early phase (0-5 minutes) and a late phase (15-50 minutes). There was no significant difference between untreated and saline-injected mice.

Systemic administration of ACEA-1011, a novel NMDA receptor/glycine site antagonist, 30 minutes prior to formalin injection produced a dose-dependent antinociception in both phases of the formalin test, as

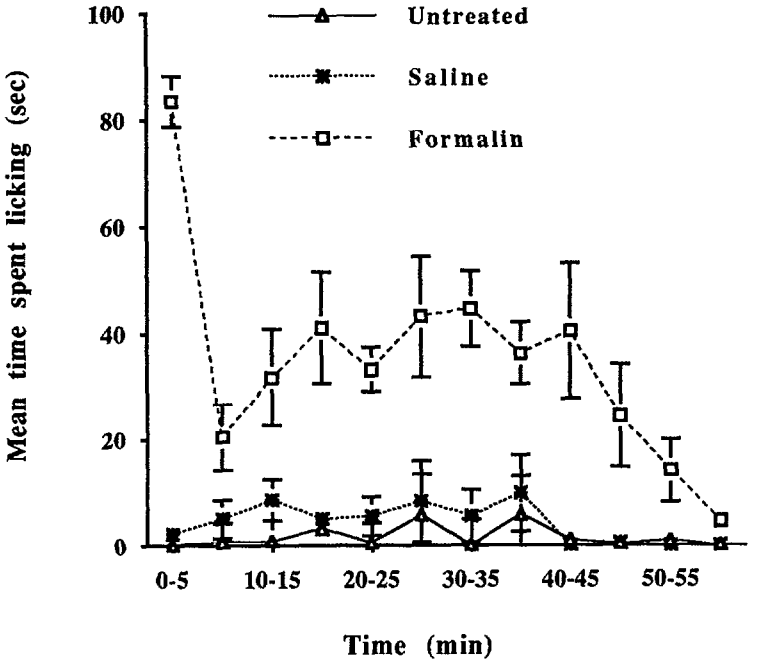

FIGURE 1. *Formalin-induced biphasic nociceptive response in male Swiss Webster mice*

compared to DMSO-treated control mice (figure 2). Pretreatment with DMSO alone had no significant effect on either of the two phases of the formalin-induced pain, compared to controls (83.5 versus 70.0 seconds for the early phase, and 36.4 versus 30.8 seconds for the late phase, respectively, for untreated mice followed by formalin versus DMSO-treated mice followed by formalin; $p > 0.05$). The analgesic ED, and 95 percent CLs for ACEA-1011 in the formalin test are shown in table 1. It is noteworthy to mention that ACEA-1011 was approximately twofold stronger in the late phase than in the early phase. Morphine, an opioid receptor agonist, produced a dose-dependent antinociception in both phases of the formalin test (figure 3). As shown in table 1, morphine decreased the mean time spent licking for the early and late phases of the formalin test, with similar analgesic ED_{50}s.

Repeated administration to mice of ACEA-1011 for 6 days produced no tolerance to the analgesic effect of the drug in the formalin test (figure 4[a]). There was a statistically significant attenuation of mean time spent licking following ACEA-1011 (20 mg/kg on the test day) in both phases

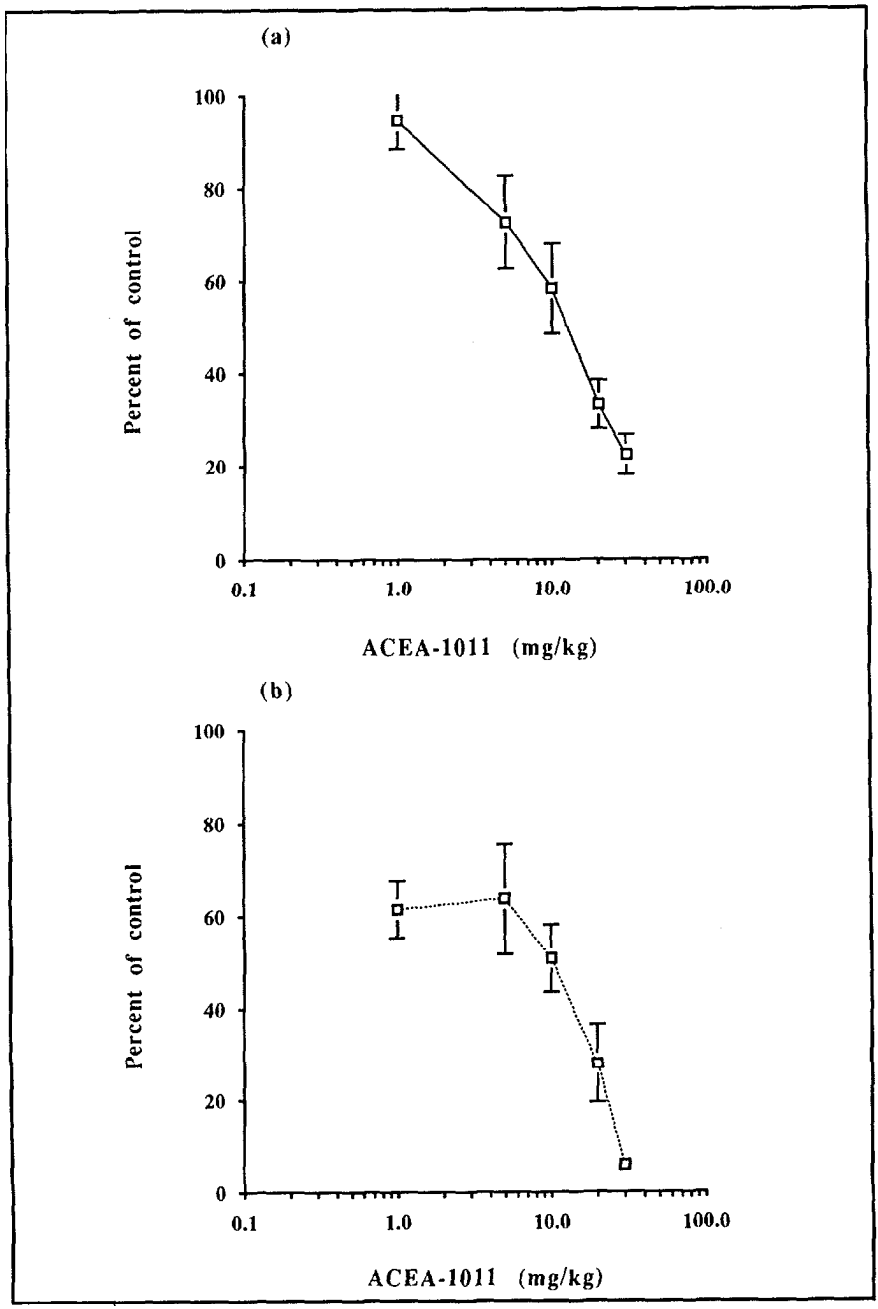

FIGURE 2. *Analgesic dose-response curves of ACEA-1011 in the early (panel a) and late (panel b) phases of the formalin test*

TABLE 1. *Analgesic ED_{50} and 95 percent confidence limits (CLs) of ACEA-1011 and morphine in the formalin test estimated by the method of Tallarida and Murray (1986)*

	ED_{50} (95% CL)	
Treatment	Early phase	Late phase
ACEA-1011	15.60(6.24-25.0)	9.45 (3.51-15.4)
Morphine	2.25 (0.60-3.90)	2.47 (0.82-4.12)

of the formalin test in both groups, ACEA-101l- and DMSO-pretreated mice, as compared to untreated (naive) controls ($F_{2,24} = 3.55$, $p < 0.02$ for the early phase; $F_{2,24} = 3.55$, $p < 0.05$ for the late phase). A one-way ANOVA followed by Newman-Keuls test revealed no statistically significant differences between ACEA-1011- and DMSO-pretreated mice in any of the phases of the formalin test ($p > 0.05$). In contrast, morphine administration for 6 days produced a significant degree of tolerance to the analgesic effect of the drug in both phases of the formalin test (figure 4[b]). Morphine was effective only in attenuating the mean time spent licking in saline-pretreated mice, as compared to morphine-pretreated mice. There was no significant difference between the mean time spent licking in morphine-pretreated (i.e., tolerant) mice and untreated (i.e., naive) mice (figure 4[b], $p > 0.05$; compare untreated group with morphine pretreated group). A one-way ANOVA followed by Newman-Keuls test revealed a significant degree of tolerance to morphine in morphine-pretreated mice, as compared to saline-pretreated mice in both the early ($F_{2,28} = 26.15$, $p < 0.02$) and the late ($F_{2,28} = 5.65$, $p < 0.05$) phases of the formalin test.

DISCUSSION

The main finding of the present study is that ACEA-1011, a novel NMDA receptor/glycine site antagonist, and morphine, an opioid receptor agonist, both produced a dose-dependent antinociception in the formalin test. Chronic exposure to morphine-produced tolerance; however, tolerance did not develop to the analgesic effect of ACEA-1011. The

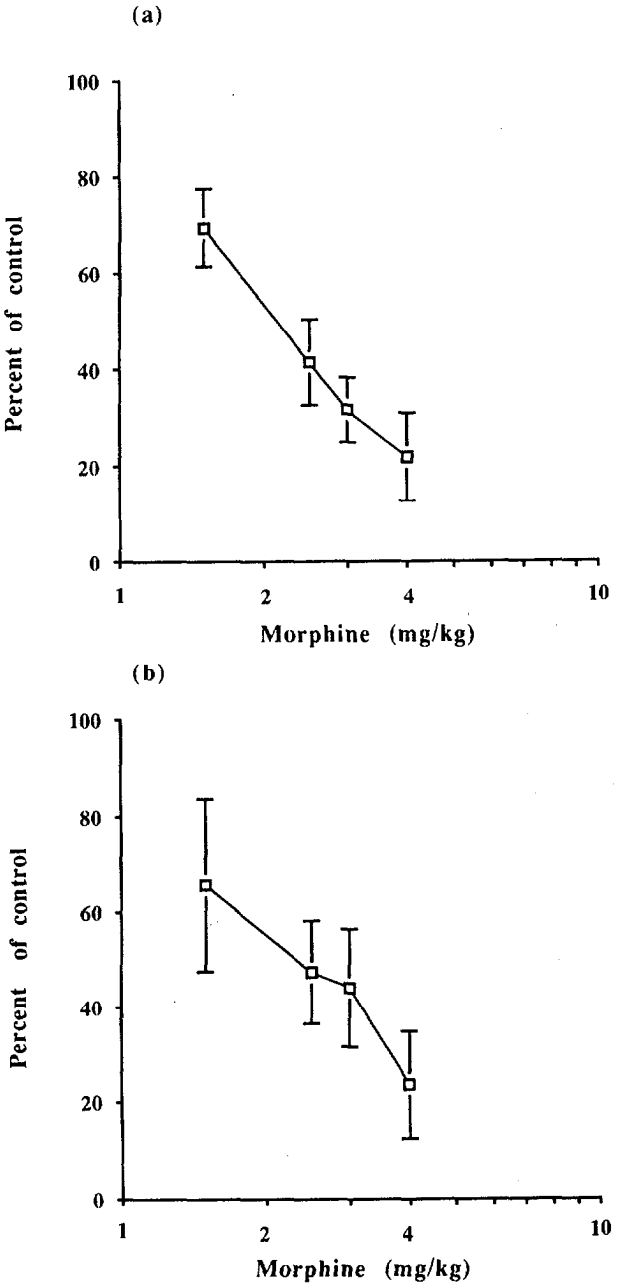

FIGURE 3. *Analgesic dose-response curves of morphine in the early (panel a) and late (panel b) phases of the formalin test*

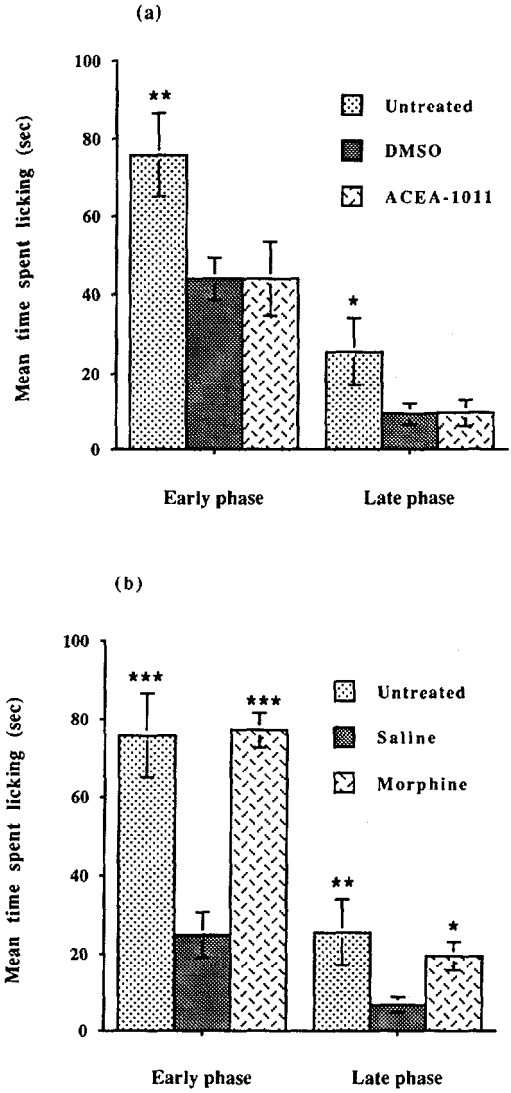

FIGURE 4. *Development of tolerance to the analgesic effects of ACEA-1011 (panel a) and morphine (panel b) in the formalin test. In panel a, *, **, and *** indicate a significant difference from DMSO- and ACEA-1011 -treated groups at $p < 0.05$ and$p < 0.02$, respectively. In panel b, *, **, and *** indicate a significant difference from saline-treated group at$p < 0.05$, $p < 0.02$, and $p < 0.01$, respectively.*

results of these studies suggest that antagonists of the NMDA receptor/ glycine site produce antinociception in the formalin test without development of tolerance.

Microinjections of formalin into the dorsal surface of the right hind paw of mice produced vigorous licking or biting of the injected paw, as reported by other investigators (Hunskaar et al. 1985; Rosland et al. 1990; Shibata et al. 1989; Vaccarino et al. 1993). Morphine decreased the mean time spent licking in a dose-related manner. Morphine was equally effective in both early and late phases of the formalin test. This is indicative of a morphine-sensitive mechanism involved in both phases of the formalin-induced nociception. Chronic administration of morphine for 6 days produced a significant degree of tolerance in the formalin test, which suggests that tolerance to morphine can be demonstrated using a tonic pain model. Thus, chronic tolerance is not a phenomenon exclusively related to the phasic pain models, such as the tail flick or hot plate tests.

ACEA-1011 produced a dose-dependent antinociceptive effect in both phases of the formalin test. The analgesic activity of ACEA-1011 in the formalin test strongly supports the results of both electrophysiological (Dickenson and Ayder 1991) and in vivo behavioral studies (Elliott et al. 1991; Millan and Seguin 1993; Murray et al. 1991; Nasstrom et al. 1992; Vaccarino et al. 1993), suggesting a modulatory role for the NMDA receptor in the control of tonic pain. The higher potency of ACEA-1011 in the late phase of the formalin test, as compared to the early phase, suggests a greater involvement of the NMDA receptor in the late phase of the formalin-induced tonic pain that is consistent with the results of electrophysiological studies (Dickenson and Ayder 199 1; Haley et al. 1990). Unlike morphine, chronic exposure to ACEA-1011 did not produce tolerance to the antinociceptive effect of the drug in either of the two phases of the formalin test. This suggests that blockade of the NMDA receptor/glycine site produces analgesia without tolerance in the formalin test. Recently, it was reported that ACEA-1011 impairs the motor activity in mice (Lutfy et al. 1994). However, at analgesic doses, this compound has no significant effect in the rotarod test. It is difficult to disregard the involvement of motor impairment completely, yet the present data demonstrate that ACEA-1011 does not produce its analgesic effect by impairing sensorimotor performance.

Initial in vitro characterization of ACEA-1011 suggests that it predominantly is an NMDA receptor/glycine site antagonist (Lutfy et al. 1994).

It displayed approximately fortyfold higher potency in blocking NMDA receptors, as compared to non-NMDA glutamate receptors. Thus, it seems likely that ACEA-1011 inhibits the formalin-induced tonic pain largely through blockade of a glycine/NMDA receptor-mediated mechanism. The blockade of both phases of the formalin-induced nociception by ACEA-1011 suggests that this compound affects both the expression and maintenance of the central changes that occur following formalin injection, effects characteristic of a general analgesic activity rather than a local one. Interestingly, it recently was demonstrated that ACEA- 1011 and other NMDA receptor/glycine site antagonists produce analgesia following intrathecal administration in the tail flick test in mice (Lutfy et al., unpublished data).

In conclusion, the results of the present study suggest that antagonism of the NMDA receptor/glycine site produces antinociception but not tolerance in the mouse formalin-induced tonic pain model. Thus, this site is a potential target for the development of new analgesics with no tolerance-inducing effects.

REFERENCES

Abbot, F.V.; Franklin, K.B.J.; Ludwick, R.J.; and Melzack, R. Apparent lack of tolerance in the formalin test suggests different mechanism for morphine analgesia in different types of pain, *Pharmacol Biochem Behav* 15:637-640, 1981.

Abbot, F.V.; Melzack, R.; and Leber, B.F. Morphine analgesia and tolerance in the tail-flick and formalin tests: Dose-response relationships. *Pharmacol Biochem Behav* 17:1213-1219, 1982.

Battaglia, G., and Rustioni, A. Coexistence of glutamate and substance P in dorsal root ganglion neurons of the rat and monkey. *J Comp Neurol* 277:302-312, 1988.

Coderre, T.J., and Melzack, R. The contribution of excitatory amino acids to central sensitization and persistent nociception after formalin-induced tissue injury. J *Neurosci* 12:3665-3670, 1992.

Dickenson, A.H., and Ayder, E. Antagonism at the glycine site on the NMDA receptor reduces spinal nociception in the rat. *Neurosci Lett* 121:263-266, 1991.

Dickenson, A.H., and Sullivan, A.F. Differential effects of excitatory amino acid antagonists on dorsal horn nociceptive neurones in the rat. *Brain Res* 506:31-39, 1990.

Dubuisson, D., and Dennis, S.G. The formalin test: A quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. *Pain* 4:151-174, 1977.

Elliott, K.J.; Cerbone, D.J.; Foley, K.M.; and Inturrisi, C.E. NMDA receptor antagonists are antinociceptive in the mouse formalin model during acute and chronic administration. *Sot Neurosci Abstr* 17:588, 1991.

Haley, J.E.; Sullivan, A.F.; and Dickenson, A.H. Evidence for spinal N-methyl-D-aspartate receptor involvement in prolonged chemical nociception in the rat. *Brain Res* 518:218-226, 1990.

Huidobro, F.; Huidobro-Toro, J.P.; and Way, E.L. Studies on tolerance development to single dose of morphine in mice. *J Pharmacol Exp Ther* 198:318-329, 1976.

Hunskaar, S.; Fasmer, O.B.; and Hole, K. Formalin test in mice, a useful technique for evaluating mild analgesics. *J Neurosci Meth* 14:69-76, 1985.

Hunskaar, S., and Hole, K. The formalin test in mice: Dissociation between inflammatory and non-inflammatory pain. *Pain* 30:103-114, 1987.

Johnson, J.W., and.Ascher, P. Glycine potentiates the NMDA response in cultured mouse brain neurons. *Nature* 325:529-533, 1987.

Kleckner, N.W., and Dingledine, R. Requirement for glycine in activation of NMDA-receptors expressed in *Xenopus oocytes. Science* 241:835-837, 1988.

Kornetsky, C., and Bain, G. Morphine: Single-dose tolerance. *Science* 162:1011-1012, 1968.

Lufty, K.; Woodward, R.M.; Keana, J.F.W.; and Weber, E. Inhibition of clonic seizure-like excitatory effects induced by intrathecal morphine using two NMDA receptor antagonists: MK-801 and ACEA-1011. *Eur J Pharmacol* 252:261-266, 1994.

Lutfy, K., and Yoburn, B.C. The role of opioid receptor density in morphine tolerance. *J Pharmacol Exp Ther* 256:575-580, 1991.

Mayer, M.L.; Vyklicky, L., Jr.; and Clements, J. Regulation of NMDA receptor desensitization in mouse hippocampal neurons by glycine. *Nature* 338:425-427, 1989.

Millan, M.J., and Seguin, L. (+)-HA 966, a partial agonist at the glycine site coupled to NMDA receptors, blocks formalin-induced pain in mice. *Eur J Pharmacol* 238:445-447, 1993.

Murray, C.W.; Cowan, A.; and Larson, A.A. Neurokinin and NMDA antagonists (but not a kainic acid antagonist) are antinociceptive in the mouse formalin model. *Pain* 44:179-185, 1991.

Nasstrom, J.; Karlsson, U.; and Post, C. Antinociceptive actions of different classes of excitatory amino acid receptor antagonists in mice. *Eur J Pharmacol* 212:21-29, 1992.
Rosland, J.H.; Tjolsen, A.; Michle, B.; and Hole, K. The formalin test in mice: Effect of formalin concentration. Pain 42:235-242, 1990.
Shibata, M.; Ohkubo, T.; Takahashi, H.; and Inoki, R. Modified formalin test: Characteristic biphasic pain response. *Pain* 38:347-352, 1989.
Skilling, S.R.; Smullin, D.H.; and Larson, A.A. Extracellular amino acid concentrations in the dorsal spinal cord of freely moving rats following veratridine and nociceptive stimulation. *J Neurochem* 51:127-132, 1988.
Tallarida, R.J., and Murray, R.B. *Manual of Pharmacologic Calculations With Computer Programs.* New York: Spinger-Verlag, 1986.
Thomson, A.M.; Walker, V.E.; and Flynn, D.M. Glycine enhances NMDA-receptor mediated synaptic potentials in neocortical slices. *Nature* 338:422-424, 1989.
Vaccarino, A.L.; Marek, P.; Kest, B.; Weber, E.; Keana, J.F.W.; and Liebeskind, J.C. NMDA receptor antagonists, MK-801 and ACEA-1011, prevent the development of tonic pain following subcutaneous formalin. *Brain Res* 615:331-334, 1993.
Way, E.L.; Loh, H.H.; and Shen, F. Simultaneous quantitative assessment of morphine tolerance and physical dependence. *J Pharmacol Exp Ther* 167:1-8, 1969.
Woolf, C.J., and Thompson, S.W.N. The induction and maintenance of central sensitization is dependent on N-methyl-D-aspartic acid receptor activation: Implications for the treatment of post-injury pain hypersensitivity states. *Pain* 44:293-299, 1991.
Yamamoto, T., and Yaksh, T.L. Comparison of the antinociceptive effects of pre- and posttreatment with intrathecal morphine and MK801, an NMDA antagonist, on the formalin test in the rat. *Anesthesiology* 77:757-765, 1992.

ACKNOWLEDGMENT

The authors would like to express their gratitude to Dr. Bogdan Sadowski, Ik-Sung Kwon, and Linda McCauley for their comments and suggestions. They are indebted to Silvia Loyola, Huy Nguyen-Khoa, Karen Kay, Ara J. Vartanian, Julie Kwon, Stacy Tran, Roxana Diba, Peter C. Rim, Peter Doan, and Kelly Park for their excellent technical assistance. This study was supported by NIDA grant DA 06356 and by Acea Pharmaceuticals, Inc.

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Etorphine Elicits Unique Inhibitory-Agonist and Excitatory-Antagonist Actions at Opioid Receptors on Sensory Neurons: New Rationale for Improved Clinical Analgesia and Treatment of Opiate Addiction

Stanley M. Crain and Ke-Fei Shen

INTRODUCTION

The opioid alkaloid etorphine (Et), a thebaine-oripavine derivative (Bentley and Hardy 1963; Blane et al. 1967) has long been known to be > 1,000 times more potent than morphine as an analgesic in animals (Blane et al. 1967; Lange et al. 1980) and humans (Blane and Robbie 1970; Jasinski et al. 1975). Blane and Robbie (1970) reported on the successful clinical use of Et as a potent analgesic administered at 1 μg/kg for periods of several weeks to about 60 chronic cancer pain patients in England. However, subsequent use of Et primarily has been confined to veterinarians for immobilization and analgesia of large animals (Blane et al. 1967). This may have been related to concerns expressed by the World Health Organization in 1966 that Et might be "particularly liable to abuse" (Jasinski et al. 1975; World Health Organization 1966) and that it showed "no dissociation from physical dependence capacity" in single-dose abstinence-suppression assays in monkeys (Deneau and Seevers 1963, cited by Blane et al. 1967; A.E. Jacobson, personal communication, August 5, 1993). Furthermore, Jasinski and colleagues (1975) reported that Et is > 1000-fold more effective than morphine in single-dose suppression of abstinence symptoms in morphine addicts. In addition, when injected subcutaneously at low analgesic doses (ca. 25-100 ug), Et elicited "morphine-like subjective effects and euphoria" in 12 nondependent but previously opiate-addicted volunteers. They concluded that "Et is appropriately classified as a morphine-like drug with a high abuse potential" (Jasinski et al. 1975).

However, these acute tests of Et were made before concepts of multiple opioid receptor subtypes were formulated, and they therefore were based on the assumption that this opioid alkaloid had properties similar to morphine. Since Et was 1,000-fold more potent than morphine in single-dose abstinence-suppression assays, it prematurely was concluded to be 1,000-fold more addictive. In contrast, electrophysiologic studies of Et effects on sensory neurons indicate that the much more potent analgesic and single-dose abstinence-suppression effects of Et versus morphine are mediated by antagonist actions of Et at excitatory opioid receptors, in addition to its potent *agonist* action at conventional *inhibitory* opioid receptors (Shen and Crain 1994*a*, 1994*b*). Therefore, acute behavioral tests of the effects of Et are not sufficient to determine the dependence liability of Et. Indeed, to the authors' knowledge, no systematic studies have been reported demonstrating physical dependence (e.g., abstinence or naloxone-withdrawal syndrome) after *chronic* administration of Et at analgesic concentrations in vivo.

Furthermore, the authors were impressed by recent clinical reports from China indicating that the Et analog dihydroetorphine (DHE) (Bentley and Hardy 1967) had been used successfully as a potent analgesic with low-dependence liability in tens of thousands of chronic pain patients and as a novel agent for detoxifying heroin addicts in > 3,000 cases (Qin 1993; Qin et al. 1994; Wang et al. 1992*a*). In addition, morphine-dependent rats no longer showed the usual naloxone-precipitated withdrawal symptoms when tested with acute naloxone (4 mg/kg) several days after replacing chronic morphine treatment (100 mg/kg) with DHE (3 μg/kg) (Wang et al. 1992*b*). This remarkable efficacy of DHE in contrast to morphine in preventing naloxone-precipitated withdrawal symptoms appeared to be quite paradoxical since DHE-induced analgesia in naive rats is prevented or reversed readily by naloxone.

Electrophysiologic studies on sensory dorsal-root ganglion (DRG) neurons in culture provide novel cellular mechanisms that may account for these unusual and paradoxical in vivo effects of Et and DHE in contrast to morphine (Shen and Crain 1989, 1994*a*, 1994*b*). These studies led to the formulation of the hypothesis that most opioid agonists can have bimodal excitatory/inhibitory effects on neuronal functions, mediated by separate subtypes of Gs-coupled excitatory opioid receptors and Gi/Go-coupled inhibitory opioid receptors, respectively (Crain and Shen 1990, 1991; Shen and Crain 1989). The authors have been attempting to identify opioids that might have selective agonist or antagonist action at inhibitory but not excitatory opioid receptors or

selective action at excitatory but not inhibitory opioid receptors. Studies of the role of sustained activation of excitatory opioid receptor functions in mediating cellular expression of physical dependence during chronic morphine treatment (Crain and Shen 1992*a*; Shen and Crain 1992; see below) suggest that "an opioid which can selectively activate inhibitory, but not excitatory, opioid receptor functions would be a unique analgesic in vivo with high potency, low dependence liability, and useful for treatment of opioid addicts" (Shen and Cram 1994*a*).

Comparative studies of Et and DHE versus morphine effects on nociceptive types of DRG neurons now have demonstrated that, in contrast to the bimodal excitatory/inhibitory effects of morphine and most other opioid alkaloids and peptides on the co-dependent component of the action potential duration (APD) of DRG neurons, Et and DHE do, in fact, elicit only dose-dependent, naloxone-reversible inhibitory shortening of the APD of naive neurons, even at remarkably low (fM-nM) concentrations, and they similarly are effective in eliciting inhibitory effects during acute application to chronic opioid-sensitized neurons (Shen and Cram 1994*u,* 1994*b).* This lack of cross-tolerance to Et in chronic morphine-tolerant DRG neurons (Shen and Crain 1994*a*, 1994*b)* may account for the unexpected absence of cross-tolerance to Et reported by Lange and colleagues (1980, 1983) in chronic morphine-treated mice, even when the analgesic ED, for systemic morphine had increased 15- to 30-fold. Furthermore, studies of sensory neurons in culture have demonstrated that this unique selective inhibitory efficacy of Et and DHE is due not only to their potent agonist action at mu, delta, kappa, and other subtypes of inhibitory opioid receptors but also to their unexpected *antagonist* action at low (pM) concentrations on mu, delta, kappa, and other subtypes of *excitatory* opioid receptor functions (Shen and Crain 1994*b*). These in vitro studies provide new insights into the mechanism of action of Et (Blane and Robbie 1970) and DHE (Qin 1993; Qin et al. 1994; Wang et al. 1992*a)* as potent analgesics with low-dependence liability and for treatment of opiate addicts.

BIMODAL EXCITATORY/INHIBITORY EFFECTS OF MORPHINE AND MOST OPIOID ALKALOIDS AND PEPTIDES ON SENSORY NEURONS

The opioid responsiveness of DRG neurons was analyzed by measuring opioid-induced alterations in the APD of DRG perikarya in fetal mouse DRG-spinal cord explants after > 3 weeks maturation in organotypic

cultures (see details of tissue culture and electrophysiology techniques in Chalazonitis and Crain 1986; Shen and Crain 1989, 1992).

Electrophysiologic studies of the effects of opioids on nociceptive types of mouse sensory DRG neurons in tissue cultures indicate that opioids can elicit both excitatory and inhibitory modulation of the action potential. This bimodal modulation is mediated by activating putative excitatory opioid receptors (Crain and Shen 1990; Shen and Crain 1989), in addition to previously characterized inhibitory opioid receptors (Chalazonitis and Crain 1986; Mudge et al. 1979; North 1986; Werz and Macdonald 1983, 1985), on these neurons. At low (< nM) concentrations, nearly all opioids tested, including morphine, enkephalins, dynorphins, endorphins, and other specific mu, delta, and kappa opioid agonists, elicit naloxone-reversible, dose-dependent excitatory effects manifested by prolongation of the APD of DRG neurons (Cram and Shen 1990; Shen and Crain 1989) (figures 1-3). In contrast, the same opioids generally elicit inhibitory APD-shortening effects when applied at higher (ca. uM) concentrations (figures 1-3). The excitatory opioid effects on sensory neurons have been shown to be mediated by opioid receptors that are coupled via a cholera toxin (CTX)-sensitive stimulatory G protein, Gs, to adenylate cyclase/cyclic AMP (cAMP)/protein kinase A-dependent ionic conductances that prolong the APD (resembling, for example, beta-adrenergic receptors) (Chen et al. 1988; Crain and Shen 1990; Cruciani et al. 1993; Fan et al. 1991; Makman et al., in press; Shen and Cram 1989,1990*a*) (figure 2). On the other hand, inhibitory opioid effects are mediated by opioid receptors that are coupled via pertussis toxin (PTX)-sensitive inhibitory G proteins: Gi to the adenylate cyclase/cAMP system and Go to ionic conductances that shorten the APD (resembling, for example, alpha$_2$-adrenergic receptors) (Crain and Shen 1990; Gross et al. 1990; Makman et al. 1988; Miyake et al. 1989; Shen and Crain 1989) (figure 2).

Shortening by opioids of the action potential of nociceptive types of primary sensory neurons generally has been considered to be a useful model of their inhibition of calcium influx and transmitter release at presynaptic terminals in the dorsal spinal cord, thereby accounting for opioid-induced analgesia in vivo (Crain and Shen 1990; Mudge et al. 1979; North 1986; Shen and Crain 1989; Werz and Macdonald 1983, 1985). Similarly, the delayed repolarization associated with the observed opioid-induced prolongation of action potentials has been interpreted as evidence of excitatory effects of opioids on these sensory neurons that may result in enhanced calcium influx and transmitter release at

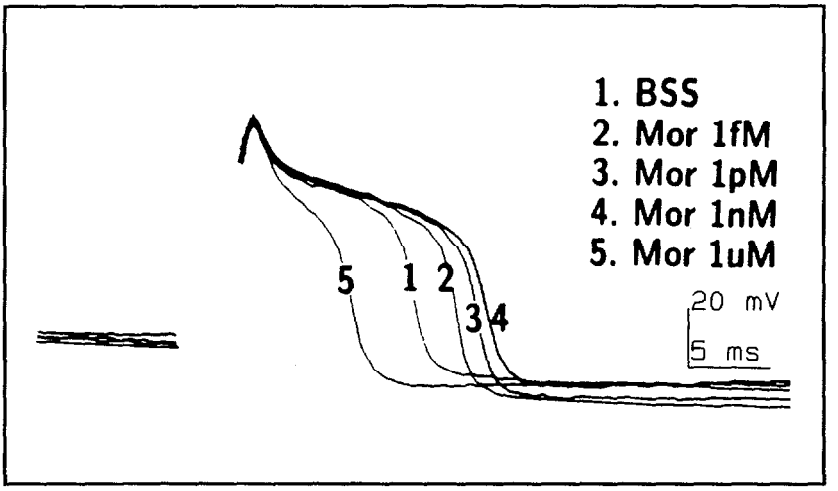

FIGURE 1. *Dual modulation by opioids of the APD of DRG neurons in orgunotypic DRG-spinal cord explant cultures. APDs are prolonged dose dependently by low (1-10 nM) concentrations of bimodally acting opioids (e.g., morphine and many mu, delta, and kappa agonists) in about 80 percent of ganglion cells tested (2-4) and shortened by higher (ca. 1 uM) concentrations in about 50 percent of cells tested (5), relative to controls (1). In this and all subsequent figures, action potentials* are *evoked* in *DRG neurons by a brief (2 msec) intracellular depolarizing current pulse. Control medium is a balanced salt solution (BSS) containing 5 mM* Ca^{2+} *and 5 mM* Ba^{2+}*. All tests were made after maturation of 14-day fetal mouse DRG neurons in DRG-spinal cord explants for* > 3 *weeks in vitro.*

NOTE: See details on tissue culture and electrophysiology techniques in Chalazonitis and Crain (1986) and Shen and Crain (1989, 1992)

presynaptic terminals (figure 2) and that could account for some types of hyperalgesia and hyperexcitatory states elicited by opioids in vivo (Crain and Shen 1990, 1992*a*; Shen and Crain 1989; Suarez-Roca and Maixner 1993).

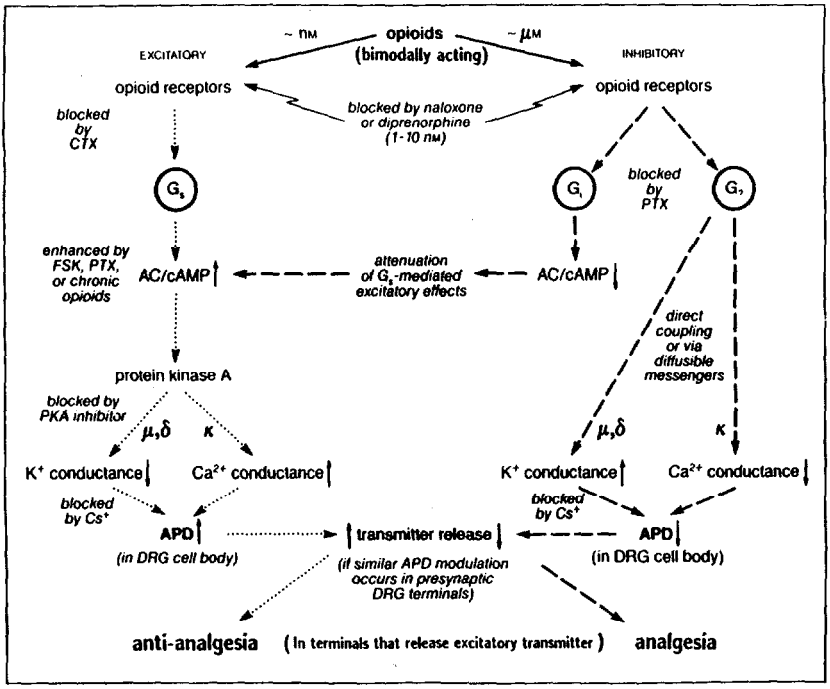

FIGURE 2. *Mechanisms that may mediate* dual modulation *by opioids of the APD and transmitter release in DRG neurons. As indicated in the lower part of the figure, opioid inhibitory APD shortening in presynaptic terminals of nociceptive DRG neurons may mediate opioid analgesia; opioid excitatory APD prolongation in other neurons in the same nociceptive network may mediate antianalgesic or hyperalgesic effects (see text).*

KEY: CTX = cholera toxin; PTX = pertussis toxin; FSK = forskolin; AC = adenylyl cyclase; PKA = protein kinase A

SOURCE: Modified from Crain and Shen (1990)

After chronic treatment of DRG neurons in culture with bimodally acting opioids (e.g., morphine or [D-Ala^2, D-Leu^5]enkephalin [DADLE] for 1 week) the cells become: (1) tolerant to the acute inhibitory APD-shortening effects of high (uM) concentrations of these opioids (Cram et al. 1988); (2) remarkably supersensitive to the excitatory APD-prolonging effects of low (< pM) as well as high concentrations of bimodally acting opioid agonists (Crain and Shen 1992*a*); and (3) responsive to nM concentrations of the opioid antagonist naloxone by

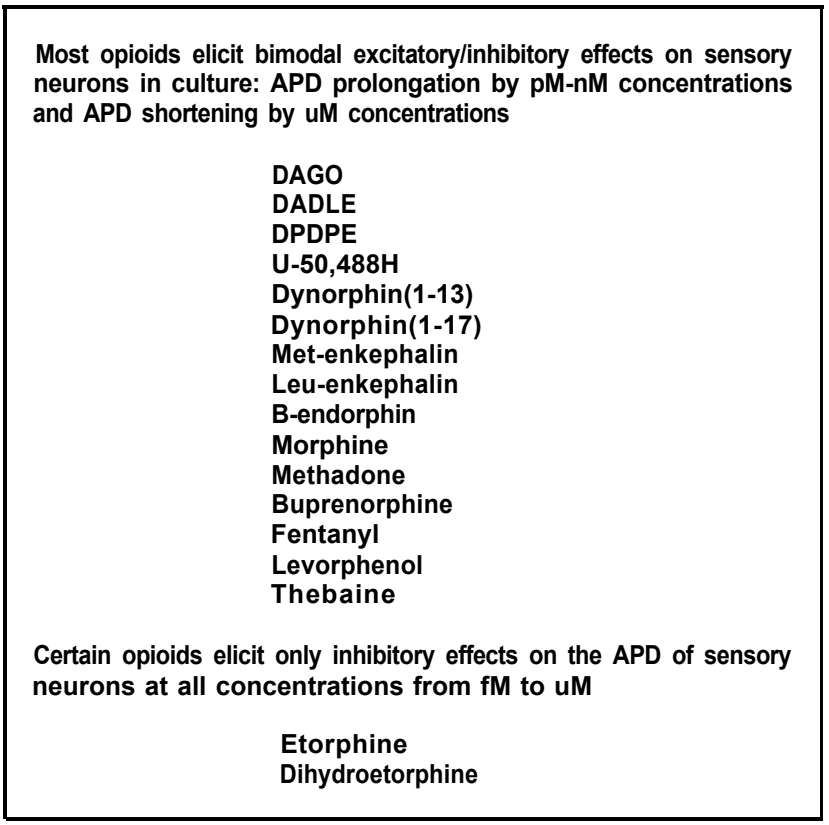

FIGURE 3. *Bimodally acting versus unimodally acting opioid agonists*

showing excitatory APD prolongation (Crain and Shen 1992*a*, 19926). As shown in records 1 and 2 of figure 4, 1 fM dynorphin prolongs the APD of a DRG neuron after chronic treatment with 1 uM DADLE for 17 days and testing in the presence of 1 uM DADLE (3-minute test). (Chronic opioid treatments generally were initiated after > 2 weeks of maturation of the fetal DRG neurons in culture.) After return to 1 uM DADLE, APD shortens toward the initial value (figure 4, record 3). APD is shortened further after raising the DADLE concentration to 10 uM (record 4). After return to 1 uM DADLE (record 5), naloxone (30 nM) *prolongs* the APD (record 6). In the presence of naloxone, raising the DADLE concentration to 10 uM no longer shortens the APD (record 7; cf. record 4). As shown in record 8, further increase in DADLE concentration to 100 uM also is ineffective (tested 2 minutes after withdrawal of naloxone). Instead, the APD is prolonged, presumably due to unmasking of opioid excitatory effects by residual naloxone

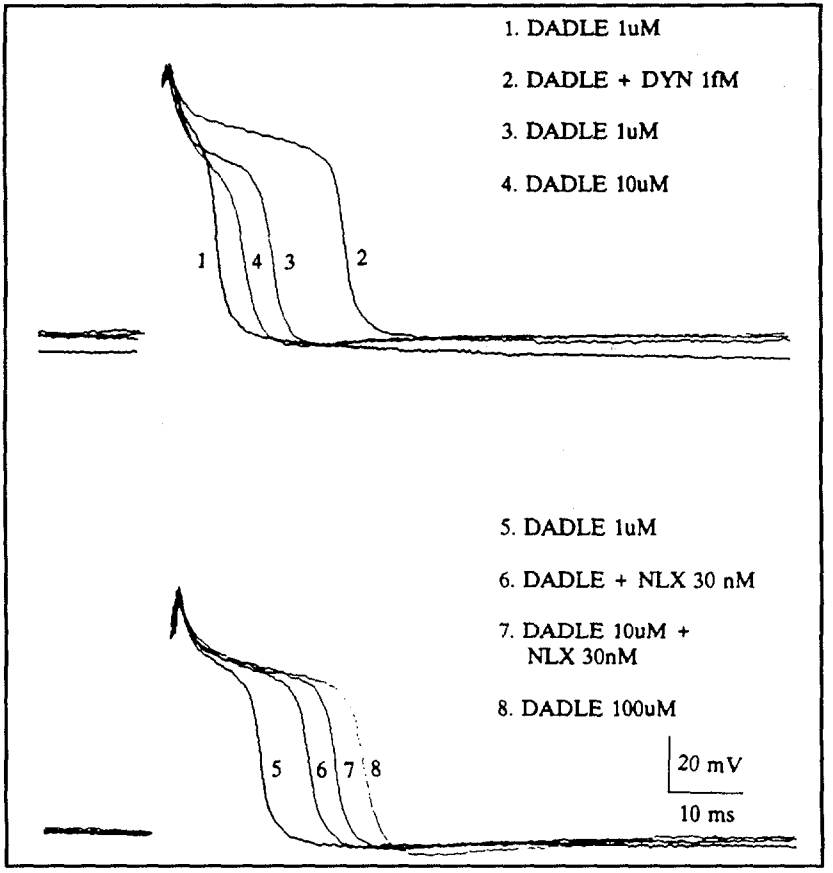

FIGURE 4. *After chronic opioid exposure, naloxone paradoxically prolongs the APD of a DRG neuron but still antagonizes opioid-induced shortening of the APD.*

KEY: DYN = dynorphin

NOTE: In this and all subsequent figures, chronic opioid treatment was initiated after > 2 weeks of maturation of the fetal DRG neurons in culture.

SOURCE: Crain and Shen (1992*a*)

antagonism of inhibitory opioid receptors (that had not become as completely desensitized as generally occurs in these chronic DADLE-treated neurons). All of these cellular manifestations of opioid tolerance and dependence (OTD) are prevented by co-administration of cholera

toxin-B subunit (CTX-B) (which selectively blocks GM1 ganglioside-regulated excitatory receptor-mediated opioid effects [Shen and Cram 1990*b;* Shen et al. 1991]) during chronic morphine or DADLE treatment (Shen and Cram 1992) (figure 5). These and related evidence led to the hypothesis that opioid physical dependence and some aspects of opioid addiction may be due to sustained activation of Gs-coupled, GM1 ganglioside- and CAMP-regulated excitatory opioid receptor functions in sensory and other types of neurons (figure 6), which render these treated cells supersensitive to the excitatory effects of extremely low concentrations of bimodally acting opioid agonists, as well as to nM concentrations of naloxone (Crain and Shen 1992*a*; Shen and Cram 1992; see also reviews by Nestler 1992; Terwilliger et al. 1991). Figure 6 illustrates the positive-feedback phosphorylation mechanism in DRG neurons chronically treated with bimodally acting opioids that may result in opioid excitatory supersensitivity by heterologous sensitization of excitatory opioid receptors. Sustained activation of excitatory opioid receptors increases adenylyl cyclase protein kinase A (PKA) (see figure 2), and glycosyltransferase activities, resulting in elevation of GM1 ganglioside and enhancement of the intrinsic efficacy of Gs-coupled excitatory opioid receptor functions (see details and references in Cram and Shen 1992*a*). Extremely low (fM-PM) concentrations of bimodally acting opioid agonists, as well as nM naloxone, then can activate these sensitized neurons, resulting in typical prolongation of the APD (following a PKA-stimulated increase in voltage-sensitive Ca^{2+} conductance or a decrease in K^+ conductance), thereby enhancing Ca^{2+} influx and transmitter release (as illustrated in figure 2). Note that sustained activation of excitatory opioid receptors during chronic opioid exposure can be blocked by: (1) CTX-A, which ADP-ribosylates Gs and attenuates agonist activation of Gs-coupled receptors (Shen and Cram 1990*a*); (2) CTX-B, which interferes with GM1 ganglioside regulation of excitatory opioid receptor functions (figure 5; Shen and Cram 1990*b*); and (3) Et, DHE or diprenorphine, all of which act directly as selective antagonists at these receptors (figures 7 and 9).

UNIQUE DUAL INHIBITORY-AGONIST AND EXCITATORY-ANTAGONIST POTENCY OF ETORPHINE (ET) AND DIHYDROETORPHINE (DHE) ON DRG NEURONS

Electrophysiologic studies of naive and chronic opioid-treated sensory DRG neurons demonstrate that Et and DHE elicit remarkably potent, naloxone-reversible, dose-dependent inhibitory APD-shortening effects,

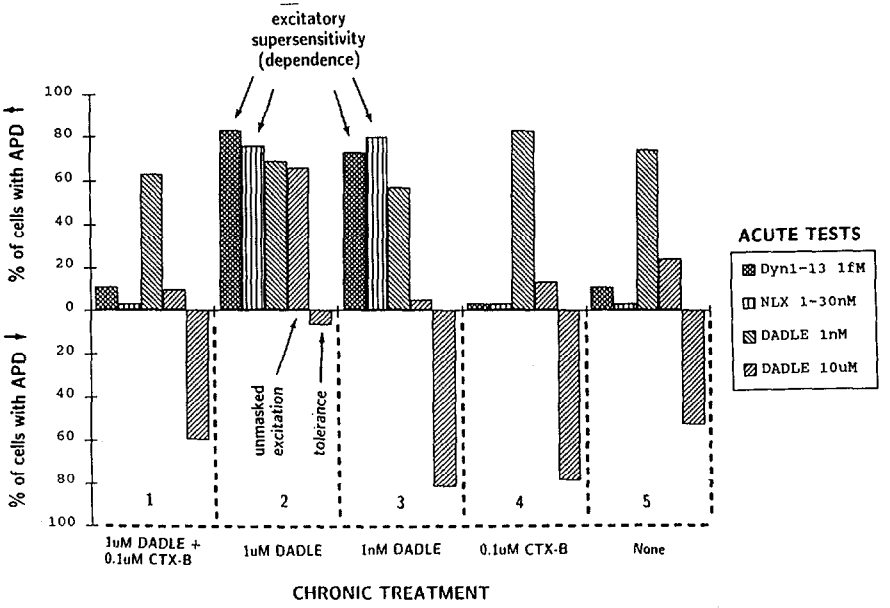

FIGURE 5. *Chronic co-administration of CTX-B with 1 uM DADLE (1-3 weeks) prevents development of supersensitivity to excitatory opioid effects and tolerance to inhibitory opioid effects in DRG neurons (column 1 versus 2). CTX-B blocks sustained activation of excitatory opioid receptor functions by binding to GM1 ganglioside regulatory site on these receptors (see figure 6). The close relationship between excitatory opioid supersensitivity (i.e., dependence) and sustained activation of excitatory opioid receptor functions is indicated by the development of similar excitatory supersensitivity after chronic treatment of DRG neurons with 1 nM instead of 1 uM DADLE (column 3 versus 2), even though no inhibitory APD shortening is evoked by nM DADLE and no tolerance develops after this treatment (cf. column 3 versus 2). DRG neurons treated with CTX-B alone show no significant alterations in opioid responsiveness, as compared to control cultures (column 4 versus 5).*

SOURCE: Shen and Crain (1992)

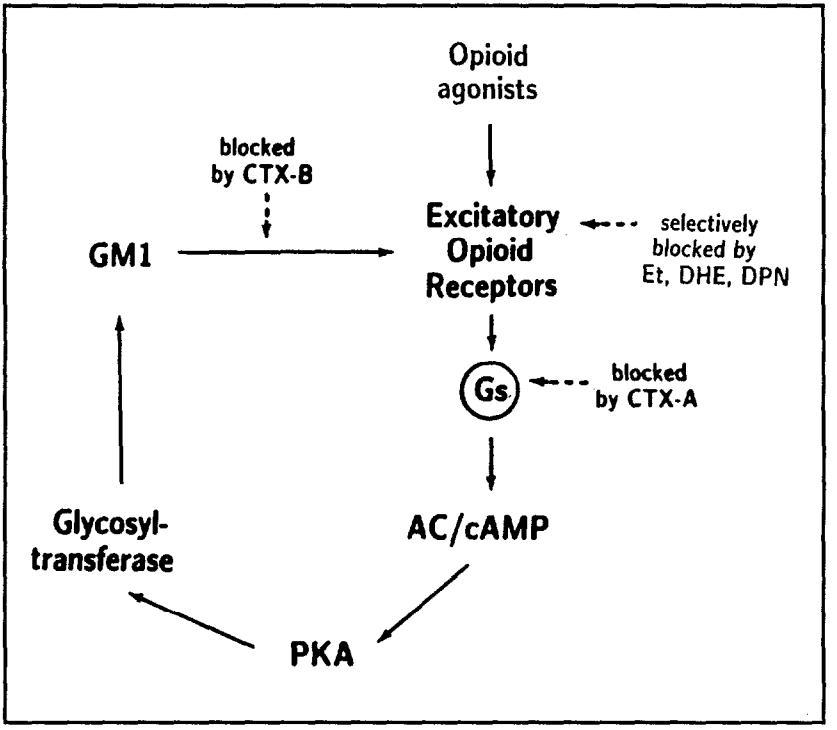

FIGURE 6. *Positive-feedback phosphorylation mechanism in DRG neurons chronically treated with bimodally acting opioids that may result in opioid excitatory supersensitivity by heterologous sensitization of* excitatory *opioid receptors.*

KEY: AC = adenylyl cyclase; DPN = diprenorphine; PKA = protein kinase A; DHE = dihydroetorphine

SOURCE: Modified from Cram and Shen (1992*a*); Shen and Cram (1992)

even at low (pM-nM) concentrations (figures 7 and 8) where morphine and all other opioid agonists so far tested elicit excitatory APD-prolonging effects (figures 1-3,7, and 8) (Shen and Crain 1994*a*, 1994*b*). As shown in records 1-5 of figure 7(a), the APD is not altered by a bath perfusion with 1 fM Et but is shortened progressively in 1 pM, 1 nM, and 1 uM concentrations (5-minute test periods). APD returns to the control

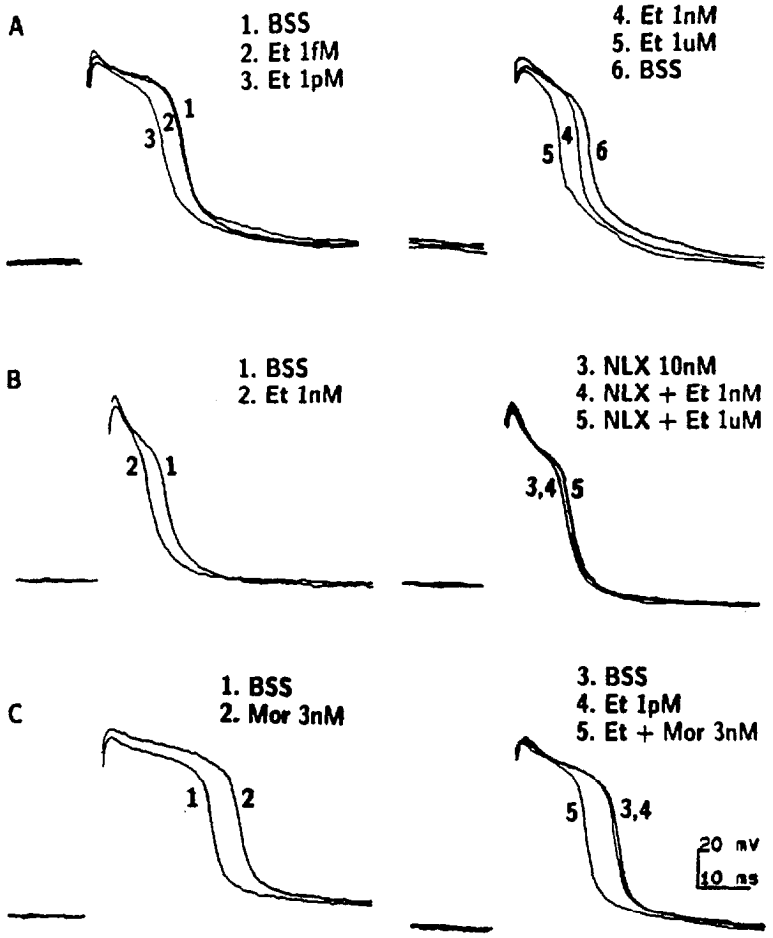

FIGURE 7. *Acute application of low (pM-nM) concentrations of Et to naive DRG neurons elicits dose-dependent, naloxone-reversible inhibitory shortening of the APD. In contrast, dynorphin and many other bimodally acting opioid agonists (e.g., morphine and DADLE) elicit excitatory APD prolongation at these low concentrations (see figure 8) that can be blocked selectively by < pM levels of Et or diprenorphine (see figure 9).*

KEY: NLX = naloxone; Mor = morphine

NOTE: Shen and Crain (1994*b*) includes more complete data and statistics on these acute tests with Et and DHE.

SOURCE: Shen and Crain (1994*b*)

value after transfer to the balanced salt solution (BSS) (record 6; 9-minute test). Figure 7(b) shows the records from another DRG neuron where the APD is shortened by application of 1 nM Et (record 2; 2-minute test) and then returns to the control value after transfer to 10 nM naloxone (record 3). APD is no longer shortened by 1 nM (record 4) or even 1 uM Et (record 5) when co-perfused with 10 nM naloxone (5-minute test periods). Figure 7(c) shows records from another DRG neuron where the APD is prolonged by application of 3 nM morphine (record 2) and then returns to the control value by 5 minutes after washout (record 3). Application of 1 pM Et does not alter the APD (record 4). Interestingly, the APD is no longer prolonged by 3 nM morphine when co-perfused with 1 pM etorphine and, instead, is shortened markedly (to a degree that would require a much higher morphine concentration in the absence of Et) (record 5). Similar results were obtained by pretreatment with 1 pM diprenorphine (see figure 9). These results indicate that Et and DHE are potent agonists at inhibitory opioid receptors (figures 7[a] and 7[b]) and remarkably potent antagonists at excitatory opioid receptors on DRG neurons (figures 7[c] and 9). Furthermore, although the universal opioid receptor antagonist diprenorphine (Paterson et al. 1983) blocks both inhibitory and excitatory opioid receptor-mediated functions in DRG neurons at nM concentrations, this opioid acts as a selective antagonist at excitatory opioid receptors when tested at lower (ca. PM) concentrations (figure 9). During application of low (pM) concentrations of Et, DHE, or diprenorphine to DRG neurons, the excitatory APD-prolonging effects of pM-nM concentrations of bimodally acting opioid agonists are blocked selectively, and potent inhibitory APD-shortening effects are unmasked (figures 7[c] and 9; Shen and Crain 1994*b*). Records 1-4 of figure 9(a) indicate that the APD of a DRG neuron is prolonged progressively by sequential bath perfusions with 3 fM, 3 pM, and 3 nM morphine. The APD of this cell is shortened only slightly after increasing the morphine concentration to 3 uM (record 5). Although acute application of uM concentrations of morphine generally shortens the APD of naive DRG neurons in these cultures and fM morphine generally does not prolong the APD, the neurons in this batch of cultures were more sensitive to the excitatory effects of morphine and were more tolerant to opioid inhibitory effects. Furthermore, the sequential increases in morphine concentration utilized to obtain dose-response data in the present study may have resulted in development of an acute tolerance to opioid inhibitory effects that was attenuated when the tests were repeated in the presence of pM diprenorphine (see below). After transfer to BSS (record 6), the APD is shortened slightly during pretreatment for 17 minutes with 1 pM

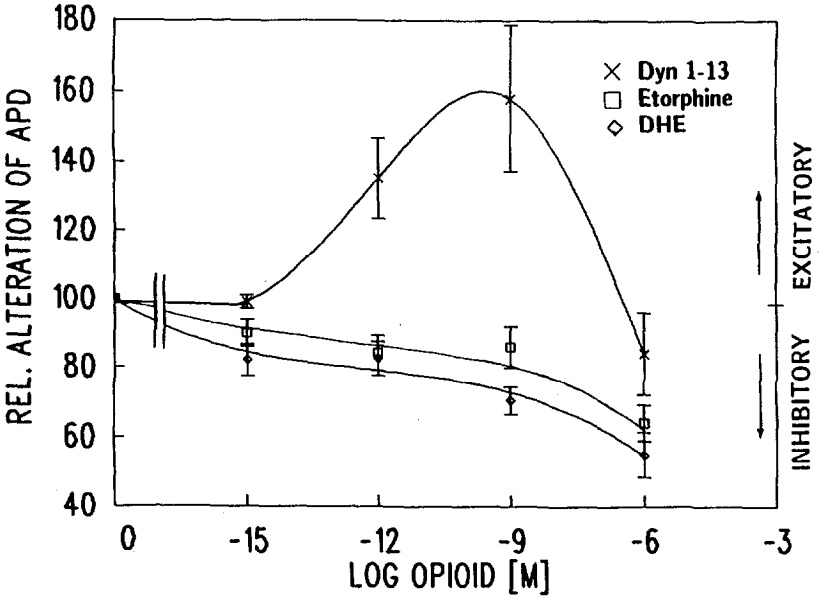

FIGURE 6. *Dose-response curves show that Et(□) and DHE (◇) elicit only inhibitory dose-dependent shortening of the APD of DRG neurons at all concentrations tested (fM-uM). In contrast, Dyn (X) (as well as morphine and other bimodally acting opioids) elicits dose-dependent excitatory APD prolongation at low concentrations (fM-nM) and generally requires much higher concentrations (ca. 0.1-1 uM) to shorten the APD, thereby resulting in a 'bell-shaped" dose-response curve. Data were obtained from 11 neurons for the Et tests, 13 for the DHE tests, and 35 for the dynorphin tests; 5, 8, and 9 neurons were, tested (as in figure 7) with all four concentrations of Et, DHE, and dynorphin, respectively (from to uM). For sequential dose-response data on the same neuron, the lowest concentrations (e.g., 1 fM) were applied first.*

KEY: Dyn = dynorphin (1-13)

SOURCE: Shen and Crain (1994*b*)

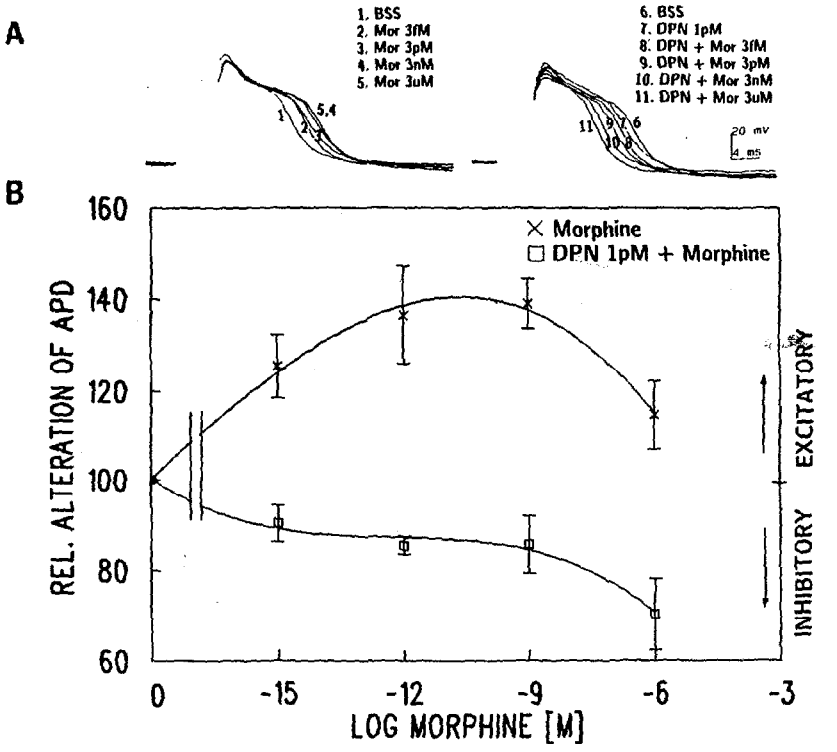

FIGURE 9. *Excitatory APD-prolonging effects elicited by morphine in DRG neurons are blocked selectively by co-administration of a low (pM) concentration of DPN, thereby unmasking potent dose-dependent inhibitory APD shortening by low concentrations of morphine.*

KEY: DPN = diprenorphine; Mor = morphine

SOURCE: Shen and Crain (19943)

diprenorphine (record 7). After the APD reached a stable value in diprenorphine, sequential applications of 3 fM, 3 pM, 3 nM, and 3 uM morphine now progressively *shorten* the APD, in contrast to the marked APD prolongation evoked by these same concentrations of morphine in the absence of diprenorphine (records 8-11; see also figure 7[c]). The dose-response curves in figure 9(b) demonstrate similar unmasking by 1 pM DPN of potent dose-dependent inhibitory APD shortening by morphine(□) in a group of DRG neurons (n = 7), all of which showed only excitatory APD prolongation responses when tested prior to introduction of diprenorphine (X). Note that the inhibitory potency of

morphine in the presence of pM diprenorphine becomes comparable to that of Et and DHE (cf. figure 8).

This unmasking of inhibitory opioid receptor-mediated effects in DRG neurons by selective antagonists at excitatory opioid receptors (see also figure 2) is consonant with the enhancement of morphine-evoked analgesia in vivo by blocking the excitatory "antianalgesic" effect of low doses of intrathecally (i.t.) injected dynorphin with antidynorphin antibodies (Fujimoto et al. 1990) or with CTX (Arts et al. 1993; Fujimoto et al. 1992). The present studies on DRG neurons suggest that the high inhibitory potencies of Et and DHE (Blane et al. 1967; Wang and Qin 1991; Wang et al. 1992*a*) are due to their unique dual opioid inhibitory receptor agonist and excitatory receptor antagonist properties, which preclude attenuation of their inhibitory effectiveness by the concomitant activation of high-affinity excitatory opioid receptors that generally occurs during application of bimodally acting opioid agonists (Shen and Crain 1994*b*). Activation of excitatory opioid receptor-mediated functions in vivo, indeed, may result in antianalgesic effects, as suggested by the studies of Arts and colleagues (1993) and Fujimoto and colleagues (1990, 1992) (see also figure 2 and below).

ACUTE APPLICATION OF ET OR DHE STILL CAN ELICIT POTENT INHIBITORY EFFECTS ON CHRONIC OPIOID-TREATEDDRGNEURONSTHATHADBECOME SUPERSENSITIVE TO OPIOID EXCITATORY EFFECTS

Acute application of fM Et to chronic uM DADLE- or morphine-treated DRG neurons (for > 1 week) still was effective in shortening the APD in 30 percent of the treated neurons (n = 23) when tested in the presence of uM DADLE or morphine (figure 10). Furthermore, pM levels of Et shortened the APD in 76 percent of the cells tested (n = 21). In contrast, these same and other chronic bimodally acting opioid-treated DRG neurons showed supersensitive excitatory APD-prolonging effects when tested with low (fM-pM) concentrations of dynorphin A(1-13) [dyn A(1-13)] before (n = 13) or after (n = 6) washout of the chronic DADLE or morphine (see additional data in Crain and Shen 1992*a*; Shen and Crain 1992). The effectiveness of Et in eliciting inhibitory APD shortening in chronic opioid-treated DRG neurons appeared to be enhanced significantly, relative to naive cells (figure 10). Dose-response

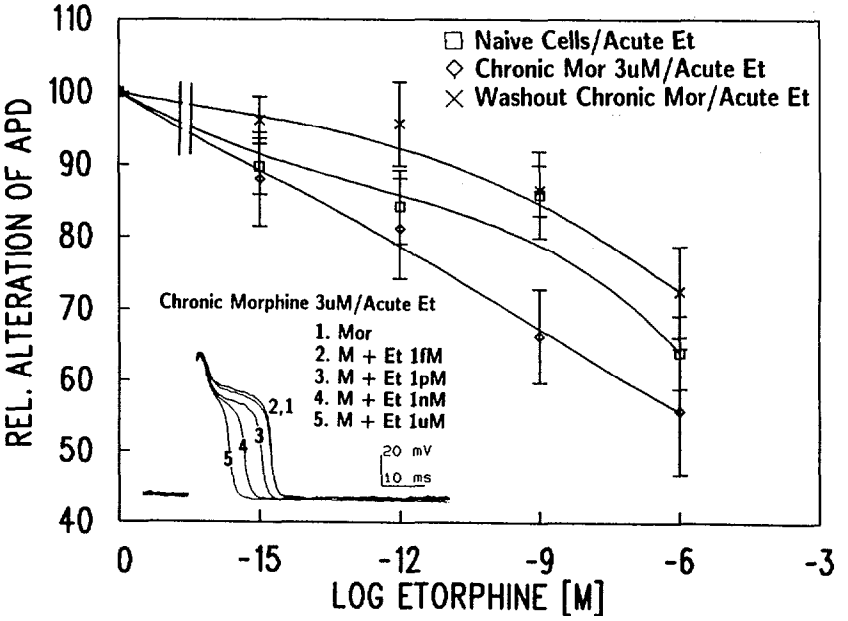

FIGURE 10. *After chronic exposure to Mor or other bimodally acting opioids, DRG neurons become supersensitive to the excitatory APD-prolonging effects of these opioids, whereas Et becomes even more effective in eliciting inhibitory shortening of* the *APD of* the same *DRG neurons when tested acutely in the presence of the chronic opioid.*

KEY: Mor = morphine

SOURCE: Shen and Crain (1994*b*)

tests of Et on chronic DADLE- or morphine-treated DRG neurons showed that the magnitude of the APD progressively was shortened when the acute Et concentration was tested sequentially from 1 fM to 1 uM in the presence of uM DADLE or morphine (figure 10). The dose-response curves in figure 10 show that, after chronic treatment of DRG neurons with 3 uM Mor (for 2-3 weeks in culture), the magnitude of APD shortening elicited by acute application of Et (◇) is enhanced markedly at all test concentrations (fM-μM; see typical records in inset), thereby shifting the dose-response curve sharply to the left, as compared to data obtained from naive DRG neurons (□). In contrast, after washout of the chronic morphine with BSS, retests of sequentially increasing

concentrations of Et from fM to uM result in less prominent APD shortening (X) comparable to or even weaker than Et effects on naive cells (□) (see statistical analyses in Shen and Crain 1994*b*). These results suggest that the apparent enhancement in inhibitory potency of Et on chronic morphine-treated neurons actually is due to unmasking of inhibitory APD-shortening effects of chronic morphine following Et-antagonist action at excitatory opioid receptors as occurs in tests on naive DRG cells (e.g., figure 7[c]). As shown in the inset of figure 10, record 1 is the action potential generated by a DRG neuron treated for 3 weeks in culture with 3 uM morphine and then tested in BSS in the presence of the chronic morphine. In the presence of 3 uM morphine, 1 fM Et does not alter the APD (record 2). However, sequential increases in the concentration of Et from 1 pM to 1 uM progressively shorten the APD in the presence of 3 uM morphine (records 3-5), whereas dynorphin dose dependently prolonged the APD of the same chronic opioid-treated cell-not shown (see figure 8).

On the other hand, after washout of the chronic morphine, acute application of Et to chronic uM opioid-treated DRG neurons no longer showed greater inhibitory effectiveness, as compared to tests on naive cells (n = 10; figure 10). These data suggest that the apparent enhancement in inhibitory effectiveness of Et (and DHE; see below) when tested during chronic exposure to bimodally acting opioid-treated DRG neurons is due to unmasking of the inhibitory actions of the chronic DADLE or morphine, resulting in a marked shift to the left in the dose-response curve for Et. Similar unmasking of inhibitory effects occurs in tests on naive neurons (e.g., figures 7[c] and 9). Acute application of DHE to chronic uM morphine-treated DRG neurons indicated that this opioid showed even greater inhibitory potency than Et: fM concentrations shortened the APD in 80 percent of the treated cells (n = 10), and pM levels were effective on *all* cells tested in the presence of the chronic opioid (n = 10).

Thus, Et and DHE show similarly remarkable effectiveness as diprenorphine in antagonizing excitatory opioid receptors, even when tested in the presence of 10^6-10^9 higher concentrations of morphine or DADLE. As a result of these unusual properties, Et and DHE showed no cross-tolerance in tests on chronic DADLE- or morphine-treated DRG neurons (Shen and Crain 1994*a*, 1994*b*), just as chronic morphine-treated mice showed no cross-tolerance to Et, even when the analgesic ED, for systemic morphine had increased 15- to 30-fold (Lange et al. 1980, 1983). Furthermore, in the latter study, Lange and colleagues (1980)

noted that 1-3 hours after removal of the implanted morphine pellet, the analgesic ED, for Et increased 8- to 10-fold, just as the enhanced inhibitory potency of Et and DHE on chronic morphine-treated versus naive DRG neurons was reduced after removal of morphine (figure 10). The absence of cross-tolerance to Et in chronic morphine-treated DRG neurons is in sharp contrast to the attenuated inhibitory effects (i.e., tolerance) and the enhanced excitatory effects (i.e., "dependence") displayed by all bimodally acting mu, delta, and kappa opioid agonists when tested acutely on these chronic opioid-treated cells (figure 4; Crain and Shen 1992*a*; Shen and Crain 1992).

ET OR DHE CAN BLOCK NALOXONE-PRECIPITATED EXCITATORY EFFECTS IN CHRONIC OPIOID-TREATED DRG NEURONS

One of the most paradoxical clinical features of "physical dependence" after chronic opiate exposure is the fact that remarkably low doses of the opioid antagonist naloxone (ca. 0.1 mg) can elicit dramatic autonomic and psychic withdrawal symptoms within a few minutes after application (see reviews by Redmond and Krystal 1984; Wikler 1980). Similarly, in neuroblastoma cell cultures, where acute opioid inhibition of adenylate cyclase and reduction in cAMP levels are reversed completely after chronic opioid treatment, application of naloxone to these "tolerant" cells elicits a dramatic increase in adenylate cyclase activity and cAMP levels (Dawson et al. 1983; Musacchio and Greenspan 1986; Sharma et al. 1975, 1977). This cell culture model of opioid dependence and withdrawal postulates that naloxone antagonizes the ongoing inhibitory influence of the chronic opioid agonist, thereby unmasking compensatory processes that had developed to counteract opioid inhibition of adenylate cyclase. However, as noted in a review by Smith and colleagues (1988), a complete model of OTD will have to account for the "apparently paradoxical observation" that in cells chronically treated with an opioid agonist opioid inhibition of cyclase disappears, indicating that opioid receptors are completely uncoupled from Gi. If so, "how could opioid antagonism result in an increase in cyclase activity?" (Smith et al. 1988).

Recent electrophysiologic studies on sensory neurons in culture may clarify the cellular mechanisms underlying these paradoxical naloxone-triggered withdrawal effects observed in vivo and in neuroblastoma cell cultures by demonstrating that acute application of low (nM) concentrations of naloxone elicits *excitatory APD-prolonging effects* in

DRG neurons, not only after chronic uM morphine or DADLE exposure (figures 4 and $11_{1,2}$) but also after *acute* treatment of naive neurons with GM1 ganglioside (Crain and Shen 1992*a*, 1992*b*). It has been proposed that both types of treatments greatly enhance the efficacy of opioid excitatory receptor functions so that even the extremely weak agonist properties of naloxone become effective in prolonging the APD of these sensitized neurons when tested at low concentrations (whereas 1nM-1uM naloxone did not alter the APD of naive DRG neurons) (Crain and Shen 1992*a*, 1992*b*). These results provide a novel cellular model to account for naloxone-precipitated withdrawal supersensitivity in opiate addicts in vivo that is based on direct excitatory effects of naloxone on supersensitive excitatory opioid receptors (Crain and Shen 1992*a*), in contrast to previous models based on naloxone antagonism of ongoing inhibitory receptor-mediated influence of the chronic opioid agonist.

It, therefore, is of great interest that acute application of remarkably low concentrations of Et (fM-nM) to chronic uM morphine- or DADLE-treated cells effectively could block naloxone-induced APD prolongation in *all* of the treated DRG neurons (n = 18; figure 11) (Shen and Crain 1994*a,* 1994*b),* thereby mimicking the potent effects of the related Et analog DHE in suppressing naloxone-evoked withdrawal symptoms in opiate addicts (Qin 1993; Qin et al. 1994; Wang et al. 1992*a*). The histogram in figure 11 shows that acute application of 1 nM naloxone to DRG neurons chronically treated with 3 uM morphine (for 1-4 weeks) prolonged the APD by about 50 percent (tested in the presence of 3 uM morphine); see also inset for records 1 and 2. In contrast, naloxone (nM-uM) does not alter the APD of naive DRG neurons (Crain and Shen 1992*a*, 1992*b*). Sequential co-perfusions with 1 fM to 1 uM Et elicit dose-dependent attenuation of the naloxone-induced APD prolongation in these treated neurons, resulting in shortening of the APD to about 70 percent of the control value in 1 uM Et. In contrast, naloxone pretreatment of naive DRG neurons blocks Et-induced APD shortening (see figure 1[b]). As illustrated in the inset of figure 11, 1 nM naloxone prolongs the APD of a DRG neuron after chronic 3 uM morphine treatment for 2 weeks and tested in the presence of 3 uM morphine (5-minute test) (record 2). Acute addition of 1 fM Et attenuates the naloxone-induced APD prolongation (record 3; 5-minute test). Increasing the concentration of Et to 1 pM almost completely blocks the naloxone-induced APD prolongation (record 4). Sequential application of 1 nM (record 5) and 1 uM Et (record 6) in the presence of morphine and naloxone results in progressive shortening of the APD well below the initial magnitude in chronic morphine (as shown in figure 10). The

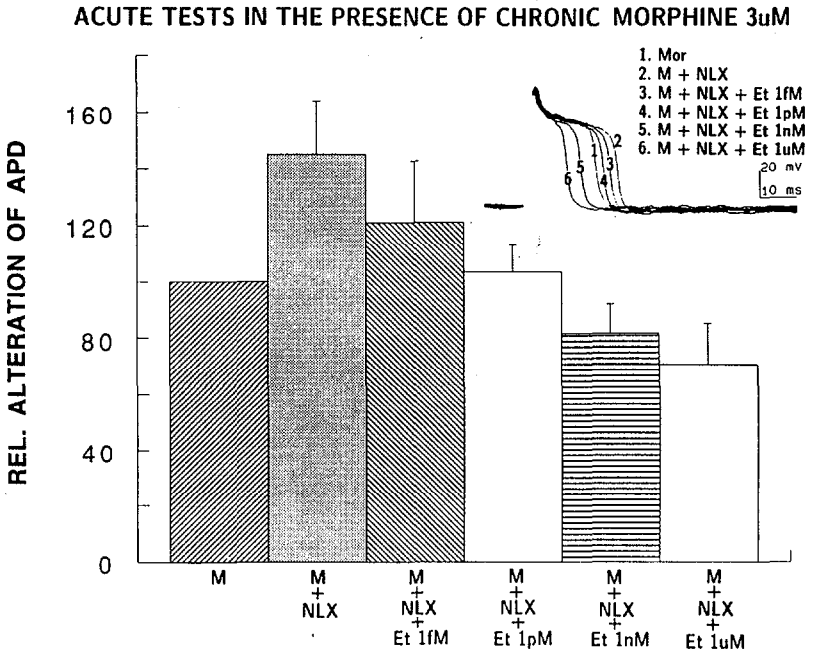

FIGURE 11. *After chronic exposure to Mor or other bimodally acting opioids acute application of low concentrations of Et dose dependently can block the excitatory APD-prolonging effects of NLX on these supersensitive DRG neurons.*

KEY: Mor = morphine; NLX = naloxone

SOURCE: Shen and Crain (1994*b*)

potent dose-dependent effects of Et and DHE in blocking naloxone-evoked APD prolongation in chronic opioid-treated DRG neurons in vitro and withdrawal syndromes in opiate addicts in vivo, therefore, may be due to the strong antagonist actions of these opioids at supersensitive excitatory opioid receptors that have become responsive to the weak agonist properties of naloxone, as well as to extremely low concentrations of bimodally acting opioid agonists (Shen and Crain 1994*b)*. This is in sharp contrast to the blockade of Et-induced APD shortening in naive DRG neurons by naloxone under conditions where it primarily acts as an antagonist at inhibitory opioid receptors (cf. figure 11 versus figure 7[3]).

CHRONIC CO-ADMINISTRATION OF ET AND MORPHINE TO DRG NEURONS PREVENTS DEVELOPMENT OF OPIOID EXCITATORY SUPERSENSITIVITY AND TOLERANCE

Co-administration of low (pM) concentrations of Et during chronic treatment of DRG neurons with uM levels of morphine was remarkably effective in preventing development of the opioid excitatory supersensitivity and tolerance that generally occurs after sustained exposure to bimodally acting opioids (Shen and Crain 1994*b*). Acute application of 1 fM dyn A(1-13) (n = 10) or 10 nM naloxone (n = 8) to DRG neurons chronically exposed to 3 uM morphine, together with 1 pM etorphine (for > 1 week), did not evoke the usual excitatory APD prolongation observed in chronic morphine-treated cells (Crain and Shen 1992*a*; Shen and Crain 1992), even when tested up to 6 hours after return to BSS. Furthermore, there was little or no evidence of tolerance to the inhibitory effects of uM morphine: 6 out of 10 cells still showed APD shortening following acute application of uM morphine similar to tests on naive DRG cells. If Et was acting simply as an agonist at inhibitory opioid receptors, one might predict that the addition of 1 pM Et, together with a 10^6-fold higher concentration of morphine, would have a negligible effect on chronic morphine-treated DRG neurons or would augment development of cellular signs of dependence. On the other hand, the observed results are accounted for readily by the potent antagonist action of Et at excitatory opioid receptors during chronic morphine treatment, thereby preventing development of opioid excitatory supersensitivity and tolerance, just as occurs during chronic opioid treatment of DRG neurons in the presence of CTX-B (figure 5; Shen and Crain 1992), which selectively interferes with GM1 ganglioside regulation of excitatory opioid receptor functions (figure 6; Shen and Crain 1990*b*; Shen et al. 1991).

CHRONIC ET-TREATED DRG NEURONS DO NOT DEVELOP OPIOID EXCITATORY SUPERSENSITIVITY OR TOLERANCE

Chronic treatment of DRG neurons with Et alone, even at a relatively high concentration (10 nM) for > 1 week in culture, also did not result in opioid excitatory supersensitivity when tested acutely with 1 fM dyn A(1-13) (26 out of 28 cells) or 1-10 nM naloxone (13 out of 14 cells), either before or after withdrawal of the chronic Et (figure 12; Shen and Crain 1994*b*). As shown in records 1 and 2 of figure 12, 1 fM dyn

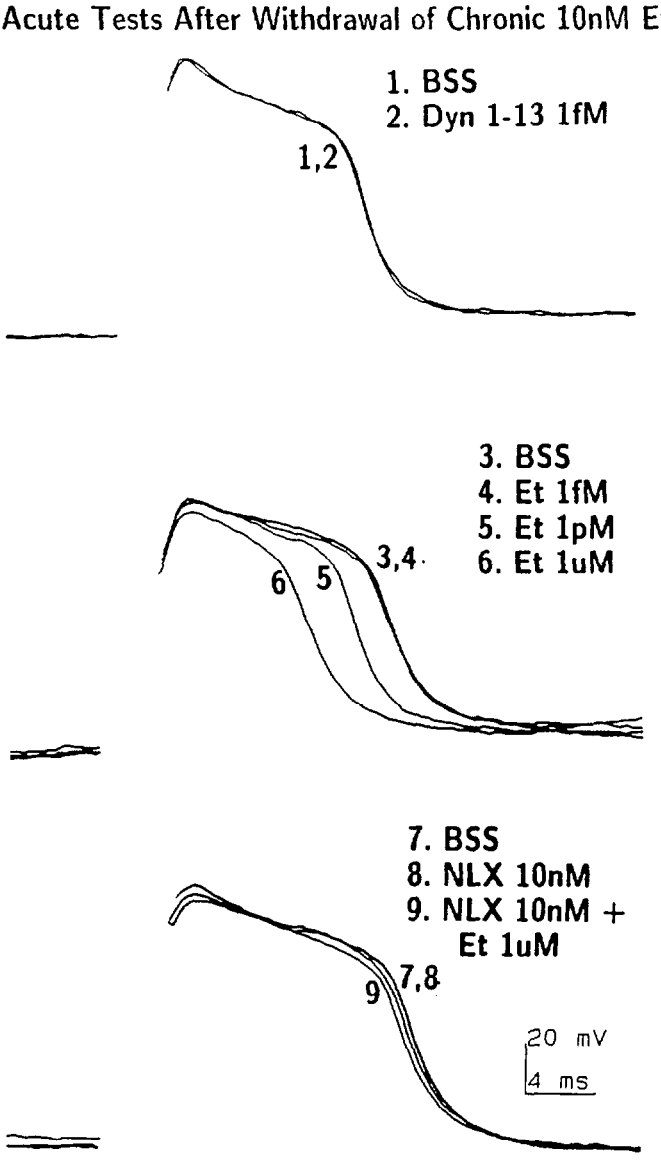

FIGURE 12. *After chronic exposure to nM Et, DRG neurons did not become supersensitive to the excitatory effects of dyn and NLX nor develop tolerance to the inhibitory effects of Et (or other opioid agonists).*

KEY: Dyn = dynorphin 1 - 13; NLX = naloxone

SOURCE: Shen and Crain (1994*b*)

does not prolong the APD of a DRG neuron chronically treated with 10 nM Et for 20 days and tested in BSS after washout of the Et. Similarly, records 7 and 8 of figure 12 show that 10 nM naloxone also was ineffective in prolonging the APD. Furthermore, after Et withdrawal for about 1 hour, 1 nM dyn A(1-13) *shortened* the APD in three cells or did not show typical APD prolongation (10 out of 11 cells), resembling the attenuation of opioid excitatory effects and unmasking of opioid inhibition observed in acute tests on naive cells after washout of low (pM) concentrations of Et (e.g., figure 7[c]). Furthermore, after washout of the chronic 10 nM Et acute application of pM, nM, and uM, Et elicited similar APD shortening (in three out of four cells tested at each concentration), as observed in naive cells (figure 12, records 5 and 6). The APD-shortening effect elicited by acute Et in these chronic Et-treated DRG neurons was antagonized by the opioid antagonist naloxone, as occurs in naive cells (figure 12, records 8 and 9; cf. record 6). Thus, chronic 10 nM Et treatment of DRG neurons did not result in the characteristic cellular signs of physical dependence and tolerance that occur after chronic exposure of these cells to morphine and other bimodally acting opioids (Crain and Shen 1992*a;* Shen and Crain 1992). Electrophysiologic data are consonant with biochemical studies reporting that the naloxone-induced adenylate cyclase "rebound response" observed in chronic morphine-treated NG108-15 neuroblastoma cell cultures (Greenspan and Musacchio 1984; Sharma et al. 1975) failed to occur when the cells were chronically treated with 10 nM Et, which markedly inhibits PGE_1-stimulated adenylate cyclase in naive NG108-15 cells (Law et al. 1983; Sharma et al. 1975; see, however, Musacchio and Greenspan 1986). On the other hand, chronic exposure of rodents to much higher concentrations of Et, in fact, did result in development of tolerance and dependence (Lange et al. 1980, 1983; Tao et al. 1987), even in the same studies where morphine-tolerant animals showed no cross-tolerance to Et (Lange et al. 1980, 1983; see below).

REMARKABLY LOW CONCENTRATIONS OF ET AND DHE CAN SHORTEN THE APD OF DRG NEURONS

The physicochemical mechanisms that account for the ability of DRG neurons to show APD shortening within a few minutes after application of such remarkably low (fM-pM) bath concentrations of Et and DHE (figures 7 and 8) and morphine (figure 9) are unclear. Although an fM solution contains only 600 molecules per cu mm, the surface charge on the DRG cell membrane might result in an increase in the local

concentration of opioid at the surface of the membrane, thereby enhancing the probability of ligand binding to receptors (see discussion by Rechling and Macdermott 1991). At any rate, the potent inhibitory effects of DHE and Et on DRG neurons are in excellent agreement with the effects of these opioids in inhibiting the muscle twitch in isolated guinea pig ileum and mouse vas deferens assays; the IC_{50} for DHE and Et in the ileum assay was 0.4 fM and 1 pM, respectively (Wang and Qin 1991). A fentanyl opioid (NIH 10741) also has been found to inhibit muscle contraction in the vas deferens assay at a concentration of 3 fM (Woods et al. 1994). In addition, serotonin has been shown to elicit comparably potent, concentration-dependent suppression of *N*-methyl-D-Aspartate (NMDA)-induced current in acutely isolated spinal dorsal horn neurons (see figure 2; dose-response curve in Murase et al. 1990), where 1 fM serotonin evokes a similar degree of inhibitory effect, as observed in the present opioid studies on DRG neurons (figures 8 and 9).

The authors previously demonstrated that these DRG neurons also display similarly striking sensitivity to the excitatory effects of very low (fM-pM) concentrations of bimodally acting opioids, including morphine and many mu, delta, and kappa opioid agonists (Crain and Shen 1992*a*; Shen and Crain 1992; Shen et al. 1991). Hundreds of control experiments with selective blocking agents (e.g., naloxone and CTX-B) and selective potentiating agents (e.g., GM1, but not GM2 or GM3, ganglioside), often tested sequentially on the *same* cells, have provided compelling evidence that these DRG neurons, in fact, are sensitive to such low opioid concentrations and that the present unexpected findings are not due to undefined technical artifacts (Crain and Shen 1992*a*; Shen and Crain 1992; Shen et al. 1991). Furthermore, the remarkably potent excitatory effects of opioids on the action potential of sensory neurons in culture are consonant with the extremely low i.t. doses of dyn A(1-17) (ca. 0.05 fmol) that elicit antianalgesic behavioral effects in normal adult mice (Fujimoto et al. 1990). In addition, 10 fM beta-endorphin elicits marked naloxone-reversible enhancement of the cytotoxic “natural killer” cell activity of human blood cells (see figure 1; dose-response curve in Mathews et al. 1983).

REMARKABLY LOW CONCENTRATIONS OF ET, DHE, AND DIPRENORPHINE CAN ANTAGONIZE EXCITATORY OPIOID RECEPTOR FUNCTIONS IN DRG NEURONS

As noted above, low fM-pM concentrations of specific opioids can elicit APD shortening or prolongation in many DRG neurons by agonist action at inhibitory or excitatory opioid receptors, respectively. Pretreatment with similarly low concentrations of Et, DHE, and diprenorphine also are remarkably effective in blocking opioid-induced APD prolongation by selective antagonist actions at mu, delta, and kappa excitatory opioid receptors. The potency of these three excitatory opioid receptor antagonists is shown clearly by their ability to unmask inhibitory opioid receptor-mediated APD-shortening effects of morphine or other bimodally acting opioids, even in the presence of 10^6-fold higher concentrations of these agonists (figures 9-11). These results suggest that Et, DHE, and diprenorphine bind with remarkably high affinity to excitatory opioid receptors. The potent antagonist action of Et at mu, delta, and kappa excitatory opioid receptors on DRG neurons is consonant with brain membrane assays demonstrating high-affinity. binding of Et (Chang et al. 1981; Kosterlitz and Paterson 1980) and DHE (Qin 1993) to conventional mu, delta, and kappa opioid receptors. Et also has been shown to activate mu, delta, and kappa inhibitory opioid receptors on various types of neurons in pharmacologic bioassays in vitro and in vivo (Magnan et al. 1982; Tao et al. 1987). Furthermore, Xu and colleagues (1992) recently have demonstrated that inhibition of the tail-flick response induced by i.t. administration of Et in mice is mediated by mu, delta, and kappa opioid receptors and is blocked by co-administration of mu, delta, and kappa antagonists.

On the other hand, in contrast to the potent antagonist action of Et at mu, delta, and kappa excitatory receptors, Et appears to be a weak partial agonist at a subtype of excitatory opioid receptors on DRG neurons that is distinct from mu, delta, and kappa subtypes. After chronic treatment of DRG neurons with PTX, which selectively inactivates opioid inhibitory receptor functions (Makman et al. 1988; Shen and Crain 1989), excitatory effects of Et are unmasked, as evidenced by prominent APD prolongation elicited by pM-nM Et. Preliminary tests indicate that the Et-induced excitatory effects in many of these neurons are not blocked by combined treatment with selective mu, delta, and kappa opioid antagonists. It will be of interest to determine if the nonmu, nondelta, and nonkappa excitatory opioid receptor that appears to mediate the excitatory effects of Et in PTX-treated DRG neurons is a subtype of the epsilon opioid

receptors, which have been shown to bind Et with high affinity (Neck et al. 1990) and which mediate antinociception induced by intracerebroventricular (but not i.t.) administration of Et (Nicholas and Li 1985; Xu et al. 1992). These in vitro studies may clarify cellular mechanisms underlying the development of tolerance and dependence in rats chronically exposed to *extremely high* concentrations of Et (Lange et al. 1980, 1983; Tao et al. 1987), which could reach the threshold for activation of Et-sensitive excitatory receptors even though mu, delta, and kappa excitatory receptors were blocked concomitantly by Et. This mechanism also might account for the moderate degree of tolerance reported in mice after chronic treatment with analgesic levels of Et (Williams et al. 1969).

DUAL OPIOID INHIBITORY-AGONIST/EXCITATORY-ANTAGONIST ACTIONS OF ET AND DHE ON DRG NEURONS IN CULTURE PROVIDE INSIGHT INTO THEIR EFFICACY AS POTENT SELECTIVE ANALGESICS AND FOR TREATMENT OF OPIATE ADDICTS

The present studies on cultured DRG neurons provide novel cellular mechanisms that may account for the potent analgesic effects of Et in vivo (Blane and Robbie 1970; Blane et al. 1967; Jasinski et al. 1975; Lange et al. 1980, 1983; Xu et al. 1992) and for the clinical efficacy of the related opioid DHE as a potent analgesic with low-dependence liability in vivo (Qin 1993; Qin et al. 1994; Wang et al. 1992*b)* and a novel agent for treatment of opiate addiction (Qin 1993; Qin et al. 1994; Wang et al. 1992*a)*. Extensive clinical usage in China demonstrated that low doses of DHE rapidly block severe opiate withdrawal symptoms in > 3,000 heroin or morphine addicts and that substitution therapy for about a week generally resulted in elimination of naloxone-precipitated withdrawal effects after DHE withdrawal (Qin 1993; Qin et al. 1994; Wang et al. 1992*a)*. The successful results obtained with DHE in detoxifying heroin and morphine addicts are in contrast to the unreliable results obtained in comparative clinical studies in China with methadone and buprenorphine (Qin 1993; Wang et al. 1992*a),* both of which showed typical bimodal excitatory as well as inhibitory effects on DRG neurons when applied acutely in preliminary electrophysiologic tests at low (< nM) versus high (uM) concentrations, respectively (figure 3). Although physical dependence was no longer detected in tests made shortly after this mode of DHE substitution treatment, long-term "psychological dependence on opiates" was observed more frequently in

populations of DHE-detoxified addicts, as compared to pain patients treated chronically with DHE (Qin 1993). The present studies on DRG neurons in vitro suggest that the paradoxically potent efficacy of low concentrations of Et and DHE in blocking naloxone-precipitated excitatory APD-prolonging effects in sensory neurons (figure 11) and withdrawal syndromes in vivo can be accounted for by the strong antagonist actions of these unique opioids at supersensitive excitatory opioid receptors that have become responsive to the weak agonist properties of naloxone, as well as to extremely low concentrations of bimodally acting opioid agonists (Shen and Crain 1994*b)*. These tissue culture studies suggest that longer term treatment of DHE-detoxified addicts with subanalgesic doses of DHE might attenuate this "psychological dependence" by selectively blocking sustained activation of sensitized excitatory (but not inhibitory) opioid receptors that may be activated chronically by *endogenous* circulating opioids (see figure 6; discussion of protracted abstinence in Crain and Shen 1992*a*).

Electrophysiologic studies on nociceptive types of sensory neurons in tissue culture provide strong support for the hypothesis that excitatory opioid receptor functions of sensory (and other) neurons may play important roles in vivo both by attenuating analgesic effects mediated by inhibitory opioid receptors and by facilitating cellular mechanisms underlying physical dependence (as well as other aversive side effects like hyperalgesia) (Crain and Shen 1990, 1992*a*; Shen and Crain 1989, 1992, 1994*a*, 1994*b*, 1994*c*). The unique properties of opioids like Et and DHE, which act both as potent agonists at inhibitory opioid receptors and antagonists at excitatory opioid receptors on sensory neurons in culture, do indeed correlate remarkably well with their much greater potency and specificity in eliciting analgesic effects in vivo, in comparison to morphine or other bimodally acting opioids. Furthermore, the present study suggests that bimodally acting opioid analgesics may be rendered more potent in reducing pain and less likely to evoke clinically undesirable side effects and dependence liability by using them in combination with a selective excitatory opioid receptor antagonist (e.g., low concentrations of Et, DHE, or diprenorphine). The effects of opioids on the action potential of nociceptive types of sensory neurons in organotypic cultures provide, therefore, an extremely sensitive and reliable in vitro bioassay to identify selective excitatory opioid receptor antagonists and selective inhibitory opioid receptor agonists that may result in more potent and specific analgesia and improved treatment of opiate dependence or addiction.

REFERENCES

Arts, K.S.; Fujimoto, J.M.; and Crain, S.M. Inhibition of the antianalgesic action of dynorphin A in mice by cholera toxin. *Pharmacol Biochem Behav* 46:623-628, 1993.

Bentley, K.W., and Hardy, D.G. New potent analgesics in the morphine series. *Proc Chem Sot* p. 220, 1963.

Bentley, K.W., and Hardy, D.G. Novel analgesics and molecular rearrangements in the morphine-thebaine group: III. Alcohols of the 6,14-endo-Ethenotetrahydrooripavine series and derived analogs of n-allylnormorphine and -norcodeine. *J Am Chem Soc* 89:3281-3292, 1967.

Blane, G.F.; Boura, A.L.A.; Fitzgerald, A.E.; and Lister, R.E. Actions of etorphine hydrochloride (M99): A potent morphine-like agent. *Br J Pharmacol Chemother* 30:11-22, 1967.

Blane, G.F., and Robbie, D.S. Trial of etorphine hydrochloride (M99 Reckitt) in carcinoma pain, preliminary report. *Proc Br Pharmacol Soc* 20:252-253P, 1970.

Chalazonitis, A., and Crain, S.M. Maturation of opioid sensitivity of fetal mouse dorsal-root ganglion neuron perikarya in organotypic cultures: Regulation by spinal cord. *Neuroscience* 17:1181-1198, 1986.

Chang, K.J.; Hazum, E.; and Cuatrecasas, P. Novel opiate binding sites selective for benzomorphan drugs (enkephalins, morphine, diprenorphine, kappa and delta receptors). *Proc Natl Acad Sci U S A* 78:4141-4145, 1981.

Chen, G.-G.; Chalazonitis, A.; Shen, K.-F.; and Crain, S.M. Inhibitor of cyclic AMP-dependent protein kinase blocks opioid-induced prolongation of the action potential of mouse sensory ganglion neuron in dissociated cell culture. *Brain Res* 462:372-377, 1988.

Crain, S.M., and Shen, K.-F. Opioids can evoke direct receptor-mediated excitatory effects on sensory neurons. *Trends Pharmacol Sci* 11:77-81, 1990.

Crain, S.M., and Shen, K.-F. Opioids can evoke direct receptor-mediated excitatory as well as inhibitory effects on sensory neuron action potentials. In: Harris, L.S., ed. *Problems of Drug Dependence.* National Institute on Drug Abuse Research Monograph 105. DHHS Pub. No. (ADM)B1-1753. Washington, DC: Supt. of Docs., U.S. Govt. Print. Off., 1991. pp. 34-39.

Crain, S.M., and Shen, K.-F. After chronic opioid exposure sensory neurons become supersensitive to the excitatory effects of opioid agonists and antagonists as occurs after acute elevation of GM1 ganglioside. *Brain Res* 575:13-24, 1992*a*.

Crain, S.M., and Shen, K.-F. After GM1 ganglioside treatment of sensory neurons naloxone paradoxically prolongs the action potential but still antagonizes opioid inhibition. *J Pharmacol Exp Ther* 260:182-186, 1992*b*.

Crain, S.M.; Shen, K.-F.; and Chalazonitis, A. Opioids excite rather than inhibit sensory neurons after chronic opioid exposure of spinal cord-ganglion cultures. *Brain Res* 455:99-109, 1988.

Cruciani, R.A.; Dvorkin, B.; Morris, S.A.; Crain, S.M.; and Makman, M.H. Direct coupling of opioid receptors to both Gs and Gi proteins in F-l 1 neuroblastoma X sensory neuron hybrid cells. *Proc Natl Acad Sci U S A* 90:3019-3023, 1993.

Dawson, G.; McLawhon, R.W.; and Scheideler, M.A. The mechanism of action of opiates and other neuroactive agents. In: Pfeiffer, S.E., ed. *Application of Tissue Culture to Neurobiology.* Boca Raton, FL: CRC Press, 1983. pp. 89-114.

Deneau, G.A., and Seevers, M.H. Evaluation of new compounds for morphine-like physical dependence in the Rhesus monkey. In: *Minutes of 26th Meeting of Committee on Problems of Drug Dependence.* Addendum 1. 1963. p. 3.

Fan, S.F.; Shen, K.-F.; and Crain, S.M. Opioids at low concentration decrease openings of K^+ channels in sensory ganglion neurons. *Brain Res* 558:166-170, 1991.

Fujimoto, J.M.; Arts, K.S.; Rady, J. J.; and Tseng, L.F. Spinal dynorphin A(l-17): Possible mediation of antianalgesic action. *Neurophamzacology* 29:609-617, 1990.

Fujimoto, J.M.; Arts, K.S.; Crain, S.M. Cholera toxin (CTX) diminishes the antianalgesic action of spinal dynorphin A in mice. *FASEB J* 6:A1302, 1992.

Greenspan, D.L., and Musacchio, J.M. The effect of tolerance on opiate dependence in the NG 108-15 model system. *Neuropeptides* 5:41-44, 1984.

Gross, R.A.; Moises, H.C.; Uhler, M.D.; and Macdonald, R.L. Dynorphin A and CAMP-dependent protein kinase independently regulate neuronal calcium currents. *Proc Natl Acad Sci U S A* 87:7025-7029, 1990.

Jasinski, D.R.; Griffith, J.D.; and Carr, C.B. Etorphine in man: 1. Subjective effects and suppression of morphine abstinence. *Clin Pharmacol Ther* 17:267-272, 1975.

Kosterlitz, H.W., and Paterson, S.J. Characterization of opioid receptors in nervous tissue. *Proc R Soc Land* [Biol] 210:113-122, 1980.

Lange, D.G.; Fujimoto, J.M.; Fuhrman-Lane, C.L.; and Wang, R.I.H. Unidirectional non-cross tolerance to etorphine in morphine-tolerant mice and role of the blood-brain barrier. *Toxicol Appl Pharmacol* 54:177-186, 1980.

Lange, D-G.; Roerig, C.S.; Fujimoto, J.M.; and Bussel, L.W. Withdrawal tolerance and unidirectional non-cross-tolerance in narcotic pellet-implanted mice. *J Pharmacol Exp Ther* 224:13-20, 1983.

Law, P.Y.; Horn, D.S.; and Loh, H.H. Opiate regulation of adenosine 3':5' cyclic monophosphate level in neuroblastoma X glioma NG 108-15 hybrid cells. *Mol Pharmacol* 23:26-35, 1983.

Magnan, J.; Paterson, S.J.; Tavani, A.; and Kosterlitz, H.W. The binding spectrum of narcotic analgesic drugs with different agonist and antagonist properties. *Naunyn Schmiedebergs Arch Pharmacol* 319:197-205, 1982.

Makman, M.H.; Dvorkin, B.; and Crain, S.M. Modulation of adenylate cyclase activity of mouse spinal cord-ganglion explants by opioids, serotonin and pertussis toxin. *Brain Res* 445:303-313, 1988.

Makman, M.H.; Dvorkin, B.; and Crain, S.M. Opioid receptor-mediated stimulation of adenylate cyclase activity of dorsal root ganglion-spinal cord explants by dynorphin and levorphanol. *Brain Res,* in press.

Mathews, P.M.; Groelich, C.J.; Sibbitt, W.L., Jr.; and Bankhurst, A.D. Enhancement of natural cytotoxicity by B-endorphin. *J Immunol* 130:1658-1662, 1983.

Miyake, M.J.; Christie, M.J.; and North, R.A. Single potassium channels opened by opioids in rat locus cerulus neurons. *Proc Natl Acad Sci U S A* 86:3419-3422, 1989.

Mudge, A.W.; Leeman, S.E.; and Fischbach, G.D. Enkephalin inhibits release of substance P from sensory neurons in culture and decreases action potential duration. *Proc Natl Acad Sci U S A* 76:526-530, 1979.

Murase, K.; Randic, M.; Shirasaki, T.; Nakagawa, T.; and Akaike, N. Serotonin suppress N-methyl-d-aspartate responses in acutely isolated spinal dorsal horn neurons of the rat. *Brain Res* 525:84-91, 1990.

Musacchio, J.M., and Greenspan, D.L. The adenylate cyclase rebound response to naloxone in the NG108-15 cells: Effects of etorphine and other opiates. *Neurophamzacology* 25:833-837, 1986.

Nestler, E.J. Molecular mechanisms of drug addiction. *J Neurosci* 12:2439-2450, 1992.

Nicholas, P., and Li, C.H. B-endorphin-(1-27) is a naturally occurring antagonist to etorphine-induced analgesia. *Proc Natl Acad Sci U S A* 82:3178-3181, 1985.

Neck, B.; Giordans, A.L.; Cicero, T.J.; and O'Connor, L.H. Affinity of drugs and peptides for U-69,593-sensitive and -insensitive kappa opioid binding sites: The U-69,593-insensitive sites appear to be the beta-endorphin-specific epsilon receptor. *J Pharmacol Exp Ther* 254:412-419, 1990.

North, R.A. Opioid receptor types and membrane ion channels. *Trends Neurosci* 9:114-117, 1986.

Paterson, S.J.; Robson, L.E.; and Kosterlitz, H.W. Classification of opioid receptors. *Br Med Bull* 39:31-36, 1983.

Qin, B.-Y. Research advances of dihydroetorphine: From analgesia to detoxification. New Drugs *Clin Rem* 2:119-123, 1993.

Qin, B.-Y.; Wang, D.X.; and Huang, M. The application of dihydroetorphine to detoxification of heroin addicts. Supplement 1. *Regul Pept* 50:S81-S82, 1994.

Rechling, D.B., and Macdermott, A.B. Lanthanum actions on excitatory amino acid-gated currents and voltage-gated calcium currents in rat dorsal horn neurons. *J Physiol* 441:199-218, 1991.

Redmond, D.E., Jr., and Krystal, J.H. Multiple mechanisms of withdrawal from opioid drugs. *Ann Rev Neurosci* 7:443-478, 1984.

Sharma, S.K.; Klee, W.A.; and Nirenberg, M. Dual regulation of adenylate cyclase accounts for narcotic dependence and tolerance. *Proc Natl Acad Sci U S A* 72:3092-3096, 1975.

Sharma, S.K.; Klee, W.A.; and Nirenberg, M. Opiate-dependent modulation of adenylate cyclase. *Proc Natl Acad Sci U S A* 74:3365-3369,1977.

Shen, K.-F., and Crain, S.M. Dual opioid modulation of the action potential duration of mouse dorsal root ganglion neurons in culture. *Brain Res* 491:227-242, 1989.

Shen, K.-F., and Crain, S.M. Cholera toxin-A subunit blocks opioid excitatory effects on sensory neuron action potentials indicating mediation by Gs-linked opioid receptors. *Brain Res* 525:225-231, 1990*a*.

Shen, K.-F., and Crain, S.M. Cholera toxin-B subunit blocks excitatory effects of opioids on sensory neuron action potentials indicating that GM1 ganglioside may regulate Gs-linked opioid receptor functions. *Brain Res* 531:1-7, 1990*b*.

Shen, K.-F., and Crain, SM. Chronic selective activation of excitatory opioid receptor functions in sensory neurons results in opioid dependence without tolerance. *Brain Res* 597:74-83, 1992.

Shen, K.-F., and Crain, SM. Etorphine elicits unique dose-dependent inhibitory effects on the action potential of sensory neurons even at low concentrations where other opioids elicit excitatory effects. Supplement 1. *Regul Pept* 50:S79-S80, 1994*a*.

Shen, K.-F., and Crain, S.M. Antagonists at excitatory opioid receptors on sensory neurons in culture increase potency and specificity of opiate analgesics and attenuate development of tolerance/dependence. *Brain Res* 636:1286-297, 1994*b*.

Shen, K.-F., and Crain, S.M. Nerve growth factor rapidly prolongs the action potential of mature sensory neurons in culture and this effect requires activation of Gs-coupled excitatory kappa opioid receptors on these cells. *J Neurusci* 14:5570-5579, 1994*c*.

Shen, K.-F., Crain, S.M.; and Ledeen, R.W. Brief treatment of sensory ganglion neurons with GM1 ganglioside enhances the efficacy of opioid excitatory effects on the action potential. *Brain Res* 559:130-138, 1991.

Smith, A.P.; Law, P.-Y.; and Loh, H.H. Role of opioid receptors in narcotic tolerance/dependence. In: Pasternak, G.W., ed. *The Opiate Receptors. New* Jersey: Humana Press, 1988. pp. 441-485.

Suarez-Rota, H., and Maixner, W. Activation of kappa opioid receptors by U50488H and morphine enhances the release of substance P from rat trigeminal nucleus slices. *J Pharmacol Exp Ther* 264:648-653, 1993.

Tao, P.L.; Law, P.-Y.; and Loh, H.H. Decrease in delta and mu opioid receptor binding capacity in rat brain after chronic etorphine treatment. *J Pharmacol Exp Ther* 240:809-816, 1987.

Terwilliger, R.Z.; Beitner-Johnson, D.; Sevarino, K.A.; Crain, S.M.; and Nestler, E.J. A general role for adaptations in G-proteins and the cyclic AMP system in mediating the chronic actions of morphine and cocaine on neuronal function. *Brain Res* 548:100-110, 1991.

Wang, D.X., and Qin, B.Y. Effect of dihydroetorphine on contraction response of guinea pig ileum, mouse and rabbit vas deferens induced by electric field stimulation in vitro. *Acta Pharmacol Sinica* 12:420-422, 1991.

Wang, D.X.; Liu, Y.S.; and Qin, B.Y. Experimental treatment of dihydroetorphine and naloxone on morphine-dependent rats and monkeys. *Chin J Pharmacol Toxicol* 6:36-40, 1992*b*.

Wang, S.F.; Yan, D.K.; Yang, Z.; and Qin, B.Y. Clinical investigation on detoxification of heroin addiction with dihydroetorphine hydrochloride. *Chin J Clin Pharmacol* 8:106-112, 1992*a*.

Werz, M.A., and Macdonald, R.L. Opioid peptides with differential affinity for mu- and delta-receptors decrease sensory neuron calcium-dependent action potentials. *J Pharmacol Exp Ther* 227:394-402, 1983.

Werz, M.A., and Macdonald, R.L. Dynorphin and neoendorphin peptides decrease dorsal root ganglion neuron calcium dependent action potential duration. *J Pharmacol Exp Ther* 234:49-56, 1985.

Wikler, A. *Opioid Dependence.* New York: Plenum, 1980.

Williams, M. W.; Williams, C.S.; Meeks, L.A.; and Gunning, B.E. Analgesic tolerance to etorphine (M99) and morphine in the mouse. *Proc Soc Exp Biol Med* 131:97-100, 1969.

Woods, J.H.; France, C.P.; Medzihradsky, F.; Smith, C.B.; and Winger, G.D. Evaluation of new compounds for opioid activity annual report (1993). In: Harris, L.S., ed. *Problems of Drug Dependence: 1993: Proceedings of the 55th Annual Scientific Meeting, The College on Problems of Drug Dependence, Inc. Vol. I, Plenary Session Symposia and Annual Reports.* National Institute on Drug Abuse Research Monograph 140. NIH Pub. No. 94-3798. Washington, DC: Supt. of Docs., U.S. Govt. Print. Off., 1994.

World Health Organization. *WHO Expert Committee on Dependence-Producing Drugs, 15th Report: World Health Organization Technical Report. Series* No. 343. Geneva: World Health Organization, 1966. pp. 1-18.

Xu, J.Y.; Fujimoto, J.M.; and Tseng, L.F. Involvement of supraspinal epsilon and mu opiod receptors in inhibition of the tail-flick response induced by etorphine in the mouse. *J Pharmacol Exp Ther* 263:246-252, 1992.

ACKNOWLEDGMENTS

This work was supported by research grants from the National Institute on Drug Abuse (DA-02031) and the Aaron Diamond Foundation to Dr. Crain. The DRG-spinal cord cultures were prepared by Peter Vanamee in tissue culture facilities kindly provided by Dr. Murray B. Bornstein.

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The Association of Neuropathic Pain, Morphine Tolerance and Dependence, and the Translocation of Protein Kinase C

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INTRODUCTION

The last few years have seen remarkable advances in the understanding of the role of excitatory amino acids (EAA), their various receptors, and the intracellular consequences of their activation in a number of medically important phenomena. This series of studies has investigated the involvement of the *N*-methyl-D-Aspartate (NMDA) receptor and the translocation of protein kinase C (PKC) in the seemingly unrelated phenomena of neuropathic pain and tolerance and dependence to narcotic analgesic drugs. It has been demonstrated that the NMDA receptor and PKC translocation are importantly involved in neuropathic pain and morphine tolerance/dependence (MTD) and that these phenomena may be importantly interrelated.

MECHANISMS OF NEUROPATHIC PAIN

Mechanical or ischemic damage to peripheral nerves that conduct somatosensory input to the central nervous system (CNS) often result in a paradoxical painful sensation denoted as *neuropathic pain* (Maciewicz and Fields 1986). The clinical features of postinjury neuropathic pain often include continuous or spontaneous pain, radiation of pain, allodynia (i.e., responding to innocuous stimulation as if it were noxious), hyperalgesia (i.e., increased responding to noxious stimulation), and temporal summation of stimulation-evoked pain (Davar and Maciewicz 1989; Merskey 1986; Stewart and Aguayo 1984; Thomas 1984).

A valuable new animal model of painful peripheral mononeuropathy has been developed recently in the rat (Bennett and Xie 1988). A peripheral mononeuropathy is produced in this model by loosely ligating the rat's common sciatic nerve. This nerve ligation procedure results in chronic constrictive injury (CCI) of the ligated nerve and abnormal nociceptive

behaviors, including hyperalgesia to radiant heat (Bennett and Xie 1988) or mechanical paw pressure stimulation (Attal et al. 1990), allodynia following cold (5 °C) (Bennett and Xie 1988) or warm (40 and 42 °C) (Attal et al. 1990; Bennett and Xie 1988) temperature stimulation, and spontaneous guarding positions of the ligated hind paw, indicative of spontaneous pain (Attal et al. 1990; Bennett and Xie 1988). Furthermore, abnormal hind paw skin temperature due to abnormal sympathetic vasomotor activity, which often is reported in human patients with postinjury neuropathic pain, also has been demonstrated in this model (Bennett and Ochoa 1991; Wakisaka et al. 1991). Thus, most clinical features of postinjury neuropathic pain syndromes can be observed in this CCI model.

This recently developed CCI model has provided a new approach to studies of CNS mechanisms underlying postinjury neuropathic pain. A series of experiments utilizing this CCI model was carried out at the Medical College of Virginia, Virginia Commomwealth University to investigate CNS mechanisms of postinjury neuropathic pain. These studies provide strong evidence for the involvement of the NMDA receptor and PKC in excitatory central mechanisms that may underlie persistent pain resulting from constrictive nerve injury and suggest new approaches towards clinical management of postinjury neuropathic pain.

Hyperalgesia and spontaneous pain due to tissue or nerve injury have been thought to be related to abnormal peripheral input (Lindblom 1990; Wall 1988). There is evidence that sensitivity of primary afferent nociceptors increases after injury, with a resultant increase in primary nociceptive afferent discharges in response to noxious stimulation (hyperalgesia) (Bessou and Perl 1969; Campbell et al. 1979; Sato and Perl 1991). Ongoing spontaneous discharges also may originate from the severed nerve in the absence of overt peripheral stimulation and thereby contribute to spontaneous pain (Devor 1983; Govrin-Lippmann and Devor 1978; Janig 1988; Wall and Gutnick 1974). However, it is likely that the prolonged abnormal peripheral input may produce CNS alterations and lead to the development of a hyperactive state within the CNS. Such a central hyperactive state may be characterized by CNS neuronal plasticity resulting from NMDA receptor- mediated intracellular changes. These hypotheses are supported by the data derived from experiments utilizing the [^{14}C]-2-deoxyglucose ([^{14}C]-2-DG) functional activity mapping technique, behavioral and pharmacological evaluations, and [^{3}H]-phorbol-12,13-dibutyrate ([^{3}H]PDBu) binding assay (Mao et al. 1992*a,* 1992*b,* 1992*c,* 1992*d,* 1992*e,* 1992*f,* 1993; Price et al. 1991).

Elevation of Spinal Cord Spontaneous Neural Activity

Spatial patterns of spinal cord neural activity were examined in unanesthetized CCI rats with painful peripheral mononeuropathy produced by sciatic nerve ligation (Mao et al. 1992*b*; Price et al. 1991). Spinal cord neural activity was assessed 10 days after nerve ligation by using the fully quantitative [^{14}C]-2-DG technique to measure local glucose utilization rates. Since the increase in local glucose utilization is proportional to the elevation of neural activity, this [^{14}C]-2-DG technique allows simultaneous examination of both neural activity inferred from local glucose utilization and its spatial distribution in multiple spinal regions previously implicated in nociceptive processing.

CCI rats used in the experiment exhibited thermal hyperalgesia to radiant heat applied to the hind paw ipsilateral to nerve ligation, as well as behaviors indicative of spontaneous pain. Sciatic nerve ligation produced a significant increase in spinal cord metabolic activity in four sampling regions (laminae I-IV, V-VI, VII, and VIII-IX) of lumbar segments of CCI rats, compared to sham-operated rats. The pattern of increased neural activity in CCI rats presented four distinct features: (1) The spinal cord grey matter both ipsilateral and contralateral to nerve ligation exhibited substantial increases in [^{14}C]-2-DG metabolic activity, as compared to sham-operated rats; (2) this increase in metabolic activity was somatotopically specific (i.e., higher metabolic rates were observed on the side ipsilateral to nerve ligation than on the contralateral side, and higher metabolic rates were seen in the medial portion of the ipsilateral spinal cord dorsal horn than in the lateral portion); (3) the peak [^{14}C]-2-DG metabolic activity occurred in laminae V-VI of CCI rats, a region generally thought to be involved in nociceptive processing; and (4) the increase in spinal cord [^{14}C]-2-DG metabolic activity in CCI rats extended from lumbar segment L_1 to L_5 in all four sampling regions. The substantial increase in neural activity in both the ipsilateral and contralateral spinal cord that occurs over an extensive rostro-caudal area in CCI rats may represent a unique pattern of spinal cord neural activity distinct from that observed in rats exposed to acute thermal pain. This pattern of spinal cord neural activity in CCI rats may reflect possible radiation of neuropathic pain.

It is important to point out that regions with the most pronounced increases in neural activity following CCI (e.g., laminae V-VI) are those containing high concentrations of nociceptive neurons (Price 1989; Willis 1985). Moreover, the substantial increase in neural activity within these

regions occurs in the absence of overt peripheral stimulation. Thus, this increase in spontaneous neural activity within spinal cord regions that have been implicated in nociceptive processing suggests a spinal cord hyperactive state and may account for spontaneous pain behaviors and hyperalgesia in CCI rats.

Attenuation of Pain-Related Behaviors in CCI Rats by NMDA Receptor Antagonists

The central hyperactive state (increases in spinal cord spontaneous neural activity) that occurs following peripheral nerve injury is induced and maintained by abnormal peripheral input, EAA-mediated central processing, or both. The role of peripheral input has been discussed elsewhere (Mao et al. 1992*c*, 1992*f*). The involvement of EAA receptors was examined in behavioral and pharmacological experiments utilizing intrathecal (i.t.) treatment with NMDA and non-NMDA receptor antagonists (Mao et al. 1992*d*, 1992*f*).

The effects of MK-801 (a noncompetitive NMDA receptor antagonist) on thermal hyperalgesia and spontaneous pain behaviors were examined in CCI rats (Mao et al. 1992*f*). Thermal hyperalgesia to radiant heat and spontaneous pain behaviors were assessed by using a foot withdrawal test and a spontaneous pain behavior rating method, respectively. CCI rats receiving intraperitoneal (i.p.) MK-801 injections (0.03, 0.1, and 0.3 mg/kg) 4 times daily beginning 15 minutes prior to nerve ligation, (preinjury treatment) exhibited significantly less hyperalgesia (i.e., longer foot withdrawal latencies) on days 3, 5, 7, 10, and 15 after nerve ligation as compared to controls receiving saline injections. Thermal hyperalgesia also was reduced when a single MK-801 injection was given i.t. onto the spinal cord lumbar segments on day 3 after nerve ligation (postinjury treatment). This effect of postinjury MK-801 treatment was dose dependent (2.5-20 nmol) and lasted for at least 48 hours after injection. Moreover, i.t. injection of MK-801 (10 nmol) reliably lowered spontaneous pain behavior rating scores in CCI rats, indicating the attenuation of spontaneous pain (Mao et al. 1992*f*). The reduction of thermal hyperalgesia in CCI rats by i.t. injection of NMDA receptor antagonists also is observed in CCI rats 7 and 11 days after nerve ligation (Yamamoto and Yaksh 1992).

Attenuation of Pain-Related Behaviors in CCI Rats by GM1 Ganglioside

A common intracellular cascade may be initiated by differential pathological conditions, including CCI, as a result of an excessive increase in intracellular C^{++} that is triggered by NMDA or non-NMDA receptor activation (Cotman and Monaghan 1989; Mayer and Miller 1990). This intracellular cascade is associated with the Ca^{++}-dependent translocation of PKC from the cytoplasm to the plasma membrane and the possible subsequent PKC activation (Alkon and Rasmussen 1988; Costa and Rodbell 1988; Nishizuka 1989), or with an alternative pathway of PKC activation relatively independent of PKC translocation (Trilivas et al. 1991). The subsequent substrate phosphorylation by the activated PKC may produce intracellular changes leading either to neuronal plastic changes associated with increases in synaptic efficacy and early gene expression or to neuronal death (Collingridge and Singer 1990; Eccles 1983; Schulman and Lou 1989; Wilcox 1991).

As described above, neural mechanisms of postinjury neuropathic pain are likely to be related to central hyperexcitation induced by EAA-mediated neuronal plastic changes. Such postinjury excitatory central alterations potentially may be interrupted at three sites (Mao et al. 1992*c*): (1) blockade of abnormal peripheral input, (2) antagonism of excessive NMDA and non-NMDA receptor activation, and (3) interruption of EAA-mediated intracellular changes. Behavioral observations indicate that postinjury neuropathic pain behaviors in CCI rats are attenuated by either peripheral nerve block or i.t. administration of NMDA receptor antagonists, and the combination of both peripheral nerve block and NMDA receptor antagonist extends the effective duration well beyond that resulting from either treatment alone. Thus, it is likely that agents that can interrupt NMDA receptor-mediated excitatory intracellular processes also may be able to effectively attenuate neuropathic pain behaviors in CCI rats.

Gangliosides are sialylated glycosphingolipids, which have been found in many tissues but with the highest concentration in the CNS (Nagai and Iwamori 1984; Schengrund 1990). GM1 ganglioside is a major subclass of cerebral gangliosides that has a wide distribution in the CNS (Nagai and Iwamori 1984; Schengrund 1990) but also has been recently identified in subsets of rat dorsal root ganglion cells (Chou et al. 1989). Functionally, gangliosides are known to play a role in neuronal differentiation and repair in many species (Schengrund 1990), including

reduction of nerve degeneration (Kojima et al. 1984; Sabel et al. 1987), promotion of nerve regeneration by increasing neurite outgrowth and sprouting (Aldinio et al. 1984; Gorio et al. 1983; Sabel and Schneider 1988; Spoerri 1986; Yavin 1986), and the reestablishment of neuromuscular trophic controls after nerve injury (Caccia et al. 1979). Recently, the in vivo application of gangliosides has been used to modulate neuronal plasticity associated with damage to both the central and peripheral nervous systems. For example, gangliosides may reduce mortality due to ischemic brain injury (Karpiak et al. 1987*a*); facilitate functional recovery following ischemic brain injury (Karpiak et al. 1987*a*), clinical stroke (Karpiak et al. 1987*b*; Leon et al. 1990), and other cerebrovascular diseases (Bassi et al. 1984); improve impaired learning ability following bilateral lesions of the caudate nucleus (Dunbar et al. 1986); and ameliorate clinical symptoms of painful peripheral neuropathies, such as diabetic, toxic, and alcoholic neuropathies (Horowitz 1986; Massarotti 1986). Most of these abnormalities are associated with EAA-induced excitotoxicity. Thus, gangliosides may be effective in reducing pain-related behaviors resulting from peripheral nerve injury in CCI rats.

An initial study examined whether preinjury treatment with gangliosides (i.e., GA, a cerebral ganglioside mixture, or GM1, a purified monosialoganglioside) reduces thermal hyperalgesia and spontaneous pain behaviors in CCI rats (Hayes et al. 1992). CCI rats received i.p. injections of 10, 20, or 40 mg/kg of GA; 10 mg/kg of GM1; or an equal volume of saline. All injections were given daily for 2 days before surgery, the day of surgery, and for 9 days after surgery. Hyperalgesic responses to radiant heat were attenuated significantly in rats receiving GA or GM1 pretreatment. In addition, pretreatment with 40 mg/kg GA also reliably reduced spontaneous pain behavior rating scores indicative of the attenuation of spontaneous pain in CCI rats. These data suggest that this animal model of peripheral neuropathic pain is sensitive to ganglioside treatment and that there is no difference between treatments with GA and GM1.

It, therefore, is clinically relevant to examine whether neuropathic pain-related behaviors in CCI rats also are reduced by ganglioside treatment that starts after nerve injury (i.e., postinjury treatment). In order to examine this possibility, CCI rats were given multiple postinjury treatments with GM1 ganglioside (Mao et al. 1992*a,* 1992*c*). GM1 treatment (10 mg/kg) initiated i.p. 1 hour or 24 hours after injury and given once daily for the first 9 postinjury days reduced thermal

hyperalgesia of the hind paw ipsilateral to nerve ligation and lowered spontaneous pain behavior rating scores in CCI rats. Thermal hyperalgesia and spontaneous pain behavior rating scores also were decreased in a dose-dependent manner in CCI rats receiving 10 daily i.t. GM1 (10-80 nmol) treatments beginning 1 hour after nerve ligation. The central site of GM1 action 'is located at the level of the lumbar spinal cord, since i.t. injection of 20 nmol GM1 onto the cervical spinal cord did not produce any protective effect.

Decrease in Spinal Cord Spontaneous Neural Activity by GM1 Ganglioside

The attenuation of thermal hyperalgesia and spontaneous pain behaviors by gangliosides may be related to the reduction of spinal cord neural activity that underlies behavioral manifestations. If this is the case, CCI rats treated with gangliosides should show decreases in spinal cord neural activity, as measured by the [^{14}C]-2-DG functional activity mapping technique. This hypothesis is supported by the observation that postinjury treatment with GM1 (10 mg/kg, i.p.) initiated 1 hour after nerve ligation and given once daily for the first 9 postinjury days reliably reduced overall increases in spinal cord neural activity in CCI rats (Mao et al. 1992*a*). First, there was a decrease in neural activity in GM1-treated CCI rats on the side ipsilateral to nerve ligation, particularly in laminae V-VI and VII and in all four sampling regions contralateral to nerve ligation. Second, unlike the rostro-caudal extension of spinal cord neural activity seen in untreated CCI rats, there was much less rostro-caudal divergence of neural activity in GM1-treated CCI rats, as compared to untreated CCI rats. In addition, both thermal hyperalgesia and spontaneous pain behaviors were reduced in CCI rats showing decreased spinal cord neural activity following GM1 ganglioside treatments. The correlation between the attenuation of thermal hyperalgesia and spontaneous pain behaviors and the reduction of spinal cord neural activity suggests a causal relationship between elevations of spinal cord neural activity and pain-related behaviors in CCI rats.

Increase in Spinal Cord Membrane-Bound Protein Kinase C (PKC)

If PKC translocation and activation are involved in central mechanisms of postinjury neuropathic pain, one would expect to see changes of PKC translocation and activation following CCI. This possibility was

examined utilizing a quantitative [^{3}H]PDBu autoradiographic assay that measures primarily membrane-bound PKC (Mao et al. 1992*e*, 1993).

CCI rats examined 3 days after sciatic nerve ligation displayed a pattern of increased membrane-bound PKC in the lumbar spinal cord strikingly different from that of sham-operated rats. Increases in membrane-bound PKC in CCI rats occurred in spinal cord regions previously implicated in nociceptive processing (laminae I-II, III-IV, and V-VI), both ipsilateral and contralateral to sciatic nerve ligation, as compared to corresponding regions of sham-operated rats. Levels of membrane-bound PKC in CCI rats, however, were reliably higher on the side ipsilateral to nerve ligation than on the side contralateral. Moreover, the spatial distribution of the increased membrane-bound PKC in CCI rats extended rostro-caudally from L_2 to L_5. All these features of increased levels of membrane-bound PKC also were observed in CCI rats examined 10 days after nerve ligation. Since CCI rats used in the experiment exhibited nociceptive behaviors both on days 3 and 10 after CCI, changes in spinal cord levels of membrane-bound PKC are associated with pain-related behaviors in CCI rats (Mao et al. 1992*e*, 1993). Taken together with the involvement of central NMDA and non-NMDA receptor activation in induction and maintenance of postinjury neuropathic pain behaviors in CCI rats, as well as the role of PKC translocation and activation in neuronal plasticity (Collingridge and Singer 1990), the results suggest that mechanisms underlying postinjury neuropathic pain may be related to CNS neuronal plastic changes involving PKC-mediated intracellular processes.

The key features of this EAA-mediated central hyperexcitation resulting from CCI are as follows. Chronic constrictive sciatic nerve injury in CCI rats produces ongoing abnormal peripheral input that may result in the continuing release of EAAs from primary afferent terminals. The sustained release of EAAs in the spinal cord dorsal horn may result in elevated and continuous activation of NMDA and non-NMDA receptors. Consequently, the activation of ionotropic NMDA and non-NMDA receptors enables an Na^+ and, most importantly, Ca^{++} influx through ligand-gated or voltage-sensitive ion channels (Cotman and Monaghan 1989; Mayer and Miller 1990; Wilcox 1991), while the activation of metabotropic receptors results in the mobilization of Ca^{++} from intracellular stores via the G protein-stimulated inositol 1,4,5-triphosphate pathway (Cotman and Monaghan 1989; Mayer and Miller 1990). Both events raise the intracellular Ca^{++} concentration to higher (pathological) levels. The resultant PKC translocation and activation may enhance the

function of Ca^{++}-dependent PKC (Alkon and Rasmussen 1988; Costa and Rodbell 1988; Nishizuka 1989; Trilivas et al. 1991).

The activated PKC may initiate a number of intracellular changes, including those related to neuronal plastic changes (Collingridge and Singer 1990; Eccles 1983; Kaczmarek 1987; Schulman and Lou 1989; Wilcox 1991), by means of PKC-induced substrate phosphorylation. For example, PKC-mediated intracellular changes may modulate ion channel activity (Kaczmarek 1987; Numann et al. 1991; West et al. 1991) and participate in enduring increases in synaptic efficacy (Collingridge and Singer 1990; Madison et al. 1991; Olds et al. 1989). These changes may occur in CCI rats, resulting in increased neuronal excitability and a central hyperactive state. This is supported by observations that spinal cord spontaneous neural activity increases substantially following CCI (Mao et al. 1992*b*; Price et al. 1991) and that there is an elevation of background discharges of spinal cord neurons, including spinothalamic tract (STT) neurons (Lair-d and Bennett 1991; Palecek et al. 1992). The exaggerated responses to mechanical or thermal stimulation in STT neurons also have been reported in CCI rats (Palecek et al. 1992). Alternatively, PKC translocation and activation can have more severe excitotoxic consequences, including cell death (Favaron et al. 1988; Magal et al. 1990; Vaccarino et al. 1987). Similar excitotoxic processes also may occur in CCI rats since there is histological evidence that CCI induces transsynaptic degeneration of spinal cord dorsal horn neurons that presumably are inhibitory interneurons (Sugimoto et al. 1989, 1990). Both direct increase in spinal cord neural activity and possible disinhibition resulting from EAA-mediated excitotoxic effects on spinal cord inhibitory interneurons may result in central hyperexcitability following constrictive peripheral nerve injury. Thus, excitatory central mechanisms may underlie, at least partially, the pathophysiological basis of postinjury neuropathic pain syndromes.

Reduction of Spinal Cord Membrane-Bound PKC by GM1 Ganglioside

Mechanisms underlying GM1-induced reductions of abnormal nociceptive behaviors and spinal cord neural activity in CCI rats are unclear. It is unlikely that gangliosides have analgesic effects similar to those produced by conventional analgesics since GM1 does not change baseline foot withdrawal latencies. The protective effects of gangliosides may be related to one or more of their many general actions on the central and peripheral nervous systems. These include: (1) reduction of plasma

membrane fatty acid loss such as occurs after ischemic injury, resulting in increased membrane integrity (Mahadii et al. 1989); (2) functional protection of membrane-associated enzymes such as plasma membrane Na^+,K^+-ATPase and mitochondrial Mg^{2+}-ATPase (Bianchi et al. 1988; Karpiak et al. 1987*a*; Li et al. 1986; Mahadik et al. 1989); (3) action as a neurotropic factor to promote neurite outgrowth and sprouting following peripheral nerve injury (Bose et al. 1986; Karpiak et al. 1987*b*; Lombardi et al. 1988; Triban et al. 1989); and (4) maintenance of normal peripheral nerve conduction and amplitude of compound action potentials following toxic neuropathy (Di Gregorio et al. 1990; Favaro et al. 1988).

Recent evidence, however, suggests that a likely mechanism of action of gangliosides in this CCI model may result from its ability to reduce EAA-mediated CNS neuronal plastic changes and excitotoxicity. As described above, postinjury neuropathic pain is related to intracellular changes mediated by abnormal central NMDA or non-NMDA receptor activation (Mao et al. 1992*e,* 1993). It has been reported that GM1 ganglioside may attenuate in vitro NMDA or non-NMDA agonist-induced neurotoxicity in cerebellar granule cells of hypoglycemic rats (Alho et al. 1988; Facci et al. 1990*a)* and chicken retinal cells (Facci et al. 1990*b)*. It also may improve in vivo neural functions after ischemic brain injury (Leon et al. 1990). On the other hand, NMDA or non-NMDA receptor-mediated intracellular changes such as PKC translocation are shown to be associated with postinjury neuropathic pain in CCI rats (Mao et al. 1992*e*, 1993). Since gangliosides have been shown to interrupt PKC-mediated intracellular changes by interfering with PKC translocation and activation (Costa and Rodbell 1988; Favaron et al. 1988; Magal et al. 1990; Vaccarino et al. 1987) or by chelating intracellular Ca^{++} (Probst et al. 1984; Rahmann 1983), it is likely that the effects of GM1 ganglioside on thermal hyperalgesia and spontaneous pain are related to the ability of GM1 to block PKC translocation.

This possibility was explored by employing the [^{3}H]PDBu binding assay to examine changes of membrane-bound PKC in CCI rats treated with GM1 ganglioside (Mao et al. 1992*e,* 1993). Three daily treatments (days 0, 1, and 2 after nerve ligation) with GM1 ganglioside reliably reduced thermal hyperalgesia and spontaneous pain behaviors in CCI rats examined 24 hours after the last treatment (i.e., day 3 after nerve ligation). Consistent with its effects on behavioral manifestations in CCI rats, GM1 treatments potently decreased overall membrane-bound PKC from spinal segments L_1 to L_5 in CCI rats examined 24 hours after the last treatment. In contrast, neither hyperalgesia and spontaneous pain

behaviors nor levels of [^{3}H]PDBu binding were changed 7 days after the last GM1 treatment (i.e., day 10 after nerve ligation), as compared to corresponding groups of nontreated CCI rats examined on the same day. Thus, there appears to be an association between the reduction of membrane-bound PKC and the attenuation of pain-related behaviors in CCI rats following GM1 ganglioside treatment. Taken together with the reduction of spinal cord neural activity in CCI rats produced by GM1 treatment, the effects of gangliosides on neuropathic pain behaviors in CCI rats may be mediated by the reduction of spinal cord hyperexcitability resulting from the interruption of NMDA receptor-mediated intracellular changes by GM1 ganglioside treatment.

Conclusion

A large body of evidence indicates that peripheral nerve injury may lead to the development of CNS hyperactive states that are mediated by central NMDA receptor activation. PKC translocation and activation may play a critical role in the development and maintenance of CNS neuronal plastic changes resulting from excessive NMDA receptor activation. The prolonged excitatory central alterations mediated by increases in synaptic transmission and early gene expression may explain why postinjury neuropathic pain often is intractable and outlasts the period of original peripheral nerve injury.

OPIOID TOLERANCE AND DEPENDENCE (OTD)

Both tolerance to and dependence upon opioids have remained largely unexplained phenomena despite extensive interest in the underlying neural mechanisms (Collin and Cesselin 1991). A number of recent studies have indicated the involvement of the NMDA receptor in opioid tolerance (Marek et al. 1991; Trujillo and Akil 1991*a*) and dependence (Marek et al. 1991). These observations suggest that the intracellular consequences of NMDA receptor activation, such as the translocation and activation of PKC, could be critical for the development of tolerance and dependence, as they have been shown to be for other neuroplastic phenomena (Domanska Janik and Zalewska 1992; Linden et al. 1988; Mao et al. 1992*e*; Olds et al. 1989). This section reviews a number of experiments conducted to examine this hypothesis. The results indicate that the translocation of PKC from neuronal cytosol to membrane may be a critical event for the development of OTD.

Attenuation of Morphine Tolerance by Inhibition Of PKC

The effects of GM1 ganglioside on the development of tolerance to the analgesic effects of 10 mg/kg of morphine given subcutaneously (s.c.) was examined in an initial study. Analgesia was measured utilizing the tail flick test (D'Amour and Smith 1941; Mayer et al. 1971). Morphine was administered for 9 days (twice/day), either 30 minutes after saline or after varying doses (10-60 mg/kg) of i.p. GM1 ganglioside. The group of rats receiving morphine and i.p. saline for 7 days showed virtually complete tolerance to the analgesic effects of morphine. In contrast, co-administration of i.p. GM1 ganglioside with s.c. morphine reduced or eliminated the development of tolerance to morphine. GM1 ganglioside produced no obvious side effects in these animals, even at the highest doses used. The decreased tolerance in groups receiving GM1 ganglioside did not result from a direct analgesic action of GM1 ganglioside because rats receiving GM1 ganglioside alone showed no increase in tail flick latencies. Nor did the increased analgesia seen in the groups treated with GM1 ganglioside result from an acute additive effect between morphine and GM1 ganglioside since animals treated with saline and morphine on days 1 through 9 and then treated with morphine (preceded by 60 mg/kg GM1 ganglioside 30 minutes earlier on day 10) showed no increase in analgesia over day 9. This observation also indicates that GM1 ganglioside can prevent the development of tolerance but cannot reverse it, at least not with a single treatment.

The involvement of the NMDA receptor in the development of tolerance, as well as the present observation that interference with a critical intracellular consequence of NMDA receptor activation, PKC translocation, blocks the development of tolerance, suggests the possibility that these treatments are interfering with higher order associative mechanisms proposed to be involved in opioid tolerance (Collin and Cesselin 1991). In order to examine this possibility, tolerance was induced to the analgesic actions of morphine by its direct application to the spinal cord. Morphine initially produced analgesia but, after 7 days of treatment with it. saline and i.t. morphine, animals showed little analgesia. In contrast, co-administration of i.t. GM1 ganglioside (40-160 nmol) with i.t. morphine reduced or eliminated the development of tolerance to morphine. This did not result from a cumulative analgesic effect of GM1 because daily i.t. administration of 160 nm GM1 for 8 days did not reliably change baseline tail flick latencies. The action of GM1 ganglioside was not due to diffusion to the brain via either the vasculature or the cerebrospinal fluid because GM1 ganglioside

application at the level of the cervical spinal cord did not prevent the development of tolerance, even though this site is closer to the brain than the effective lumbosacral sites. These results indicate that reduction of tolerance development by GM1 ganglioside need not utilize supraspinal associative mechanisms, although the involvement of such mechanisms at supraspinal levels in other forms of tolerance development cannot be ruled out (Collin and Cesselin 1991; Trujillo and Akil 1991*b*).

Attenuation Of Morphine Dependence By Inhibition Of PKC

The development of opioid tolerance often is associated with the development of opioid dependence (Collin and Cesselin 1991). Since NMDA receptor antagonists, in addition to blocking opioid tolerance, have been shown to block the development of opioid dependence (Trujillo and Akil 1991*a*), the effect of co-administration of saline or GM1 ganglioside (10-60 mg/kg) on the development of dependence resulting from repeated (9 days/twice per day) administration of morphine (10 mg/kg, i.p.) was examined. In one test, dependence was assessed by administering the narcotic antagonist naloxone. This compound has virtually no effects when administered to naive animals but precipitates a well-known withdrawal syndrome in morphine-dependent rats (as well as in man). In this experiment, naloxone-precipitated (2 mg/kg, s.c.) jumping was measured. In dependent animals not given GM1 ganglioside, a large number of jumps are elicited by naloxone. Co-administration of GM1 ganglioside with morphine over the 9 days, however, reduced the number of jumps, and the highest dose almost completely eliminated jumping. Again, as with morphine tolerance, dependence as measured by naloxone-precipitated jumping could be reduced by co-administration of GM1 ganglioside with morphine over 9 days but could not be prevented by a single injection of GM1 ganglioside on day 10 after dependence was established. In another test possibly related to dependence, weight loss was measured over the 9 days of drug administration, Animals given morphine and saline lost significant amounts of weight over the course of the experiment, while those given morphine and GM1 ganglioside did not. Thus, as with NMDA receptor blockers, GM1 ganglioside prevents the development of at least two signs of dependence.

Tolerance-Related Increase in Spinal Cord Membrane-Bound PKC

In order to more directly examine the hypothesis that translocation of PKC is involved in the development of MTD, the effects of repeated i.t. morphine treatments alone or combined with GM1 ganglioside (160 nmol) on membrane-bound PKC in the spinal cords of rats were examined utilizing a quantitative [^{3}H]PDBu autoradiographic assay (Worley et al. 1986). Daily i.t. morphine injections (10 μg) for 8 days resulted in a reliable increase in membrane-bound PKC, compared to controls injected for 7 days with saline and given a single 10 μg i.t. dose of morphine on day 8. This increase was quite specific in that it was restricted to the superficial dorsal horn. Co-administration of GM1 ganglioside with morphine reliably reduced the increase in membrane-bound PKC seen after treatment with morphine alone. This did not result from the effects of repeated treatment with GM1 ganglioside on spinal cord PKC translocation because, in another group of rats, repeated treatment with GM1 ganglioside did not alter baseline levels of membrane-bound PKC. Thus, repeated treatments with morphine result in an increase in membrane-bound PKC, and this increase can be prevented by co-administration of GM1 ganglioside with morphine.

Implications for Neural Mechanisms of MTD

These results, combined with the results of others (Marek et al. 1991; Trujillo and Akil 199 1 a), indicate that an NMDA receptor-mediated intracellular cascade known to be associated with other forms of neural plasticity (Collingridge et al. 1992; Hayes et al. 1988; Linden et al. 1988; Mao et al. 1992*c*) is likely to be associated with the development of MTD at specific and restricted loci within the CNS. This cascade, initiated by opiate receptor activation and subsequent NMDA receptor activation, results in calcium influx, PKC translocation, and presumably subsequent phosphorylation of an as-yet-unknown membrane protein or proteins (Alkon and Rasmussen 1988; Collingridge and Singer 1990).

The association of the NMDA receptor with PKC translocation and activation now is well known. NMDA receptor activation and subsequent PKC translocation have been demonstrated to be involved with neuroplastic phenomena, such as long-term potentiation (Miyamoto and Okada 1993), hyperalgesia (see work discussed in this chapter), and neural injury (Choi 1990; Favaron et al. 1988; Vaccarino et al. 1987). Results of the work discussed in this chapter support the concept that

OTD is a subset of neuroplastic phenomena that utilize common neurobiological mechanisms. Although NMDA receptor activation and the resulting intracellular cascade have been associated with neurotoxicity (Choi 1990; Favaron et al. 1988; Vaccarino et al. 1987), it may be that sometimes this physiological sequence underlies normal adaptive responses to novel or extreme environmental events.

These results may provide insights into the details of locus and mechanism of opiate action, at least within the spinal cord dorsal horn. Opiates are known to have both presynaptic and postsynaptic inhibitory effects within the spinal cord (Yaksh 1986). Although the acute pain-inhibitory effects of opiates may involve both presynaptic and postsynaptic action, it is difficult to envision how presynaptic inhibitory effects of opiates could activate any NMDA receptor, even presynaptic ones if they exist (Smimova et al. 1993), and the subsequent intracellular cascade now known to be necessary for the development of tolerance and dependence. In the case of postsynaptic action of opiates, however, it is possible to conceptualize several mechanisms. The interaction of the opiate receptor with the NMDA receptor could occur within the same cell or could result from circuitry involving two or more neurons. The latter possibility would require that activation of the opiate receptor ultimately excite a pool of neurons containing an EAA neurotransmitter, which then is released onto NMDA receptors. Although this possibility cannot be eliminated, it seems unlikely since there are few neurons in the spinal cord dorsal horn that are excited by opiate administration in vivo (Collin and Cesselin 1991). Thus, it seems most likely that opiate and NMDA receptors interact within the same cell. Results of the present study indicate that lamina II of the spinal cord dorsal horn is a critical locus for these cells because, by far, the greatest increase in translocated PKC is seen there.

With regard to the details of the interactions of opiates, the NMDA receptor and PKC (Chen and Huang 1991) have shown in vitro that opiates initiate an increase in the glutamate-elicited inward membrane current in the trigeminal equivalent of dorsal horn cells mediated by the NMDA receptor. This activation results from a PKC-mediated removal of the magnesium blockade of the NMDA receptor (Chen and Huang 1992). Nevertheless, it is well known that opiates, at analgesic doses, produce inhibition or block excitation of dorsal horn cells (Yaksh 1986). The present data may provide some resolution of these seemingly paradoxical observations.

Figure 1 proposes a schematic model of the consequences of opiate receptor activation at a postsynaptic site in the spinal cord dorsal horn. Opiate receptor occupation by an exogenous ligand such as morphine may initiate G protein-mediated PKC translocation and activation. The involvement of a second messenger-mediated system is suggested by the observation that PKC-mediated NMDA receptor glutamate sensitization takes about 2-4 minutes to occur after application of opiates (Chen and Huang 1991). PKC activation causes a removal of the magnesium blockade of the NMDA receptor (Chen and Huang 1992). With this blockade removed, even small amounts of EAA ligands (Fox and Daw 1993) for the NMDA receptor could allow localized calcium channel opening, resulting in activation of additional PKC from the same or separate pools (Allbritton et al. 1992). This additional PKC could have effects at several sites. As shown in figure 1, a PKC-mediated alteration in gene expression may be involved since morphine tolerance at the doses utilized in these experiments is not observed until after at least 3 days of morphine injections. Treatment with GM1 ganglioside, however, is unlikely to be able to block PKC binding at a nuclear level because GM1 is thought to prevent PKC translocation at the neuronal membrane surface (Favaron et al. 1988; Vaccarino et al. 1987). Thus, it is more likely that GM1 interferes either with the second pool of PKC described above or with an additional pool of PKC that could result from a modification of gene expression. Such an action could prevent an uncoupling of the opiate receptor from an associated G protein that has been proposed as a mechanism of OTD (Collin and Cesselin 1991). That this additional translocation of PKC occurs as a result of repeated opiate administration, as compared to acute opiate administration, is supported by the authors' observation of greatly increased membrane-bound PKC after repeated morphine treatment, as compared to a single treatment.

These results are consistent with the observation that a surprisingly wide range of neurotransmitter manipulations lessen OTD (Collin and Cesselin 1991). The authors' model would suggest that any manipulation that tended to prevent NMDA receptor activation, calcium influx, or the translocation of PKC would inhibit the induction of tolerance and dependence. For example, an inhibitory transmitter such as gamma amino butylic acid might stabilize the membranes of the critical cells retarding or preventing this cascade of events. On the other hand, it also is possible that multiple mechanisms are involved in OTD and that other neurotransmitters or modulators participate in those mechanisms (Collin and Cesselin 1991).

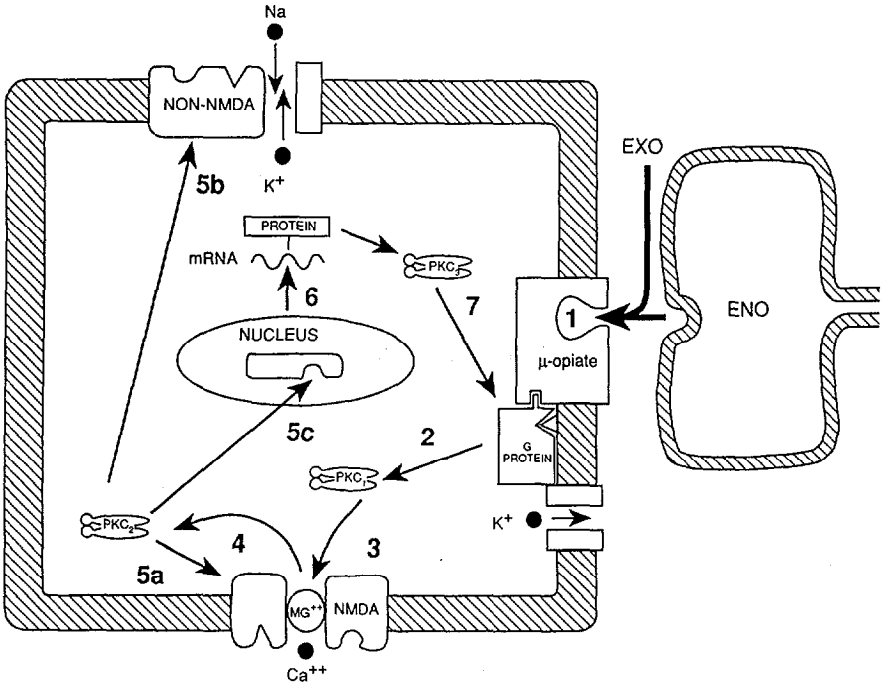

FIGURE 1. *Schematic representation of PKC involvement in OTD. Activation of a p-opiate receptor (1) initiates a second messenger-mediated PKC translocation, which (2) results in removal of the magnesium blockade from an NMDA receptor (3). The chapter's authors suggest that removal of the magnesium blockade from the NMDA receptor by this opiate action allows an influx of calcium, which (4) activates either a separate pool of PKC (PKC_2) or much greater amounts of the pool of PKC_1. There also may be intermediate steps involving the initiation of gene expression by PKC or another intracellular messenger (5c), subsequent transcription and translation (6), and the translocation of another pool of cytosolic PKC (PKC_3) to the membrane (7). This and the second pool of PKC could exert action by phosphorylation of various receptors (e.g., 5a and 5b) or by initiating a functional uncoupling of the μ-opiate receptor from a G protein, as has been suggested by others.*

Neuronal Interactions Of MTD And Neuropathic Pain

Because MTD and neuropathic pain have a number of neuronal mechanisms in common, the model in figure 1 makes some explicit predictions concerning how these phenomena should interact with each other. The proposed central mechanisms of tolerance and hyperalgesia would suggest that any manipulation that tended to activate NMDA receptors, increase intracellular calcium, or enhance PKC activity would induce opioid tolerance and hyperalgesia. If this is the case, one would expect to see a positive interaction between morphine tolerance and hyperalgesia. In particular, the development of morphine tolerance would be expected to be associated with the development of hyperalgesia, and, conversely, the condition of hyperalgesia would initiate opioid tolerance.

With regard to the possibility of tolerance-induced hyperalgesia, the authors' recent study has demonstrated that nonnaloxone-precipitated thermal hyperalgesia does develop in animals rendered tolerant to the analgesic effect of morphine (Mao et al. 1994). In this study, tolerance to the analgesic effect of morphine was developed reliably in rats following once-daily i.t. injection of 10 μg morphine sulfate for 8 consecutive days. In association with the development of morphine tolerance, hyperalgesia to radiant heat developed in these same rats. Paw withdrawal latencies were decreased reliably in morphine-tolerant rats but not in nontolerant (i.e., saline-treated) controls when tested on day 8 before the last morphine treatment and on day 10 (i.e., 48 hours after the last morphine treatment), as compared to baseline paw withdrawal latencies obtained on day 1 before any drug treatment. The development of both morphine tolerance and thermal hyperalgesia was potently prevented by i.t. co-administration of morphine with the NMDA receptor antagonist MK-801 or the PKC inhibitor GM1 ganglioside. MK-801 but not GM1 also reliably reversed thermal hyperalgesia in rats rendered tolerant to morphine when tested 30 minutes after each treatment on day 10 (48 hours after the last morphine treatment). The data strongly indicate that hyperalgesia develops in association with the development of morphine tolerance and that at least some common neural substrates (such as activation of NMDA receptors and PKC) are involved in the development of both morphine tolerance and tolerance-associated hyperalgesia.

That hyperalgesia develops in association with the development of morphine tolerance supports the idea that the decreased efficacy of opiate analgesics, following their repeated administration, may be the result of

both diminished opiate analgesic effects and the development of tolerance-associated hyperalgesia. That is, the development of tolerance to the analgesic effects of opiates may be exacerbated by the development of hyperalgesia.

Just as pain can be exacerbated by opioid tolerance, the present model predicts that opoid tolerance may be initiated or exaggerated by neuropathic pain; that is, chronic activation of the NMDA receptor by persistent pain should initiate calcium influx that should initiate the same intracellular mechanisms activated by opiate administration. This prediction is supported by the results of another of the authors' recent studies (Mao et al., submitted). In this study, the development of morphine tolerance was tested in rats with neuropathic hyperalgesia resulting from loose ligation of the rat's sciatic nerve (Bennett and Xie 1988). The hypothesis was that nerve-injured rats would exhibit less analgesia than sham-operated rats in response to a single morphine administration at 7 days after nerve ligation. At 7 days after nerve ligation, radiant heat-evoked paw withdrawal latencies were reliably reduced in nerve-injured rats (i.e., hyperalgesia), as compared to sham-operated rats. In order to make a valid comparison, pretreatment paw withdrawal latencies in nerve-injured rats were normalized by a single dose of MK-801, a procedure known to reverse hyperalgesia but not morphine tolerance, so that both groups (the nerve injury versus the sham operation) had comparable pretreatment of paw withdrawal latencies. Under this condition, a single i.t. injection of morphine produced reliably less analgesia in nerve-injured rats than in sham-operated rats. These results indicate that tolerance to the analgesic effects of morphine has developed in nerve-injured rats before the first exposure to morphine.

The data of this study have bearing on the controversy concerning the efficacy of opiates for the treatment of neuropathic pain states (Davar and Maciewicz 1989; Dubner 1991; Kupers et al. 1991; Portenoy et al. 1990). It is conceivable that the diversity of clinical response patterns to opiate treatment in neuropathic pain patients may result from varying degrees of central neuronal plastic changes, which are influenced by differences in the time course and severity of original etiological factors causing pain. Not only would such central neuronal plastic changes predispose the subject to tolerance conditions, but they also would exacerbate opioid tolerance as the treatment continues. On the other hand, it should be recognized that the degree of tolerance and dependence in these patients might never have been adequately assessed because of the progression of some chronic pain states such as neuropathic pain and cancer pain. In

particular, it is different to determine whether the need for increasing opiate doses during a treatment process results from the progression of original pain states, the development of OTD, and tolerance-associated hyperalgesia. In this sense, knowledge of commonality and interactions of neural mechanisms underlying these phenomena may provide new insights into the management of these clinical situations.

Whatever the details of the neurochemical events underlying tolerance and dependence, the present results have important implications for the treatment of chronic pain, OTD and possibly tolerance to and dependence on other drugs of abuse. These results demonstrate that GM1 ganglioside, a substance already utilized for treatment of several clinical syndromes (Rodden et al. 1991)—as well as other relatively nontoxic substances interfering with NMDA receptor activation or PKC translocation or activation—could prevent the development of tolerance to and dependence on opioids used for the treatment of chronic pain. Similarly, the development of tolerance and dependence to opioids used for other purposes might be prevented. Results indicate that a single dose of GM1 ganglioside cannot reverse tolerance and dependence. It is possible, however, that repeated doses might reverse tolerance and dependence, as has been demonstrated for the competitive NMDA receptor blocker LY274614 (Tiseo and Inturrisi 1993), making possible the treatment of at least some aspects of opiate addiction. It also is possible that the development of tolerance to and dependence on other drugs involves similar mechanisms. Such a possibility is supported by the observations that the development of tolerance to other abused substances such as alcohol (Wu et al. 1993) and cocaine (De Montis et al. 1992) can be reduced by NMDA receptor antagonists.

SUMMARY

This series of studies has investigated the involvement of the NMDA receptor and the translocation of PKC in the seemingly unrelated phenomena of neuropathic pain and tolerance and dependence to narcotic analgesic drugs. This work has demonstrated that the NMDA receptor and PKC translocation are importantly involved in neuropathic pain and morphine tolerance or dependence and that these phenomena may be importantly interrelated.

Neuropathic pain following nerve injury is a major chronic pain syndrome. Utilizing a rat model of painful peripheral mononeuropathy

produced by CCI of the sciatic nerve, the authors have investigated central mechanisms of postinjury neuropathic pain. Behavioral and pharmacological studies indicate that thermal hyperalgesia and spontaneous pain behaviors observed in this model are attenuated by treatment with NMDA receptor antagonists. A consequence of NMDA receptor activation is calcium influx, which in turn can result in translocation of PKC from cytosol to membrane. Inhibitors of intracellular PKC translocation and activation block thermal hyperalgesia and spontaneous pain behaviors after CCI and also reduce the elevated spinal cord neural activity in CCI rats. Furthermore, spinal cord levels of membrane-bound PKC reliably increase in CCI rats as a result of translocation of PKC revealed by the [^{3}H]PDBu autoradiographic assay. This increase in membrane-bound PKC is associated with postinjury neuropathic pain behaviors in CCI rats and both pain-related behaviors and membrane-bound PKC are reduced potently by GM1 ganglioside.

Because MTD has been shown to involve the NMDA receptor, the role of PKC in these phenomena also has been examined. The authors have shown that the development of MTD is reduced greatly by co-administration with morphine of inhibitors of PKC translocation and activation. Rats made tolerant to i.t. administration of morphine showed increased membrane-bound PKC in the superficial layers (laminae I and II) of the spinal cord dorsal horn but not in deeper layers. This increase is prevented by co-administration with morphine of inhibitors of PKC translocation and activation. These results indicate that the translocation and activation of PKC may be a critical step in the development of MTD. In addition, the authors have shown that: (1) the induction of morphine tolerance results in hyperalgesia, a symptom of neuropathic pain, and this hyperalgesia can be prevented by NMDA receptor antagonists and GM1 ganglioside, and (2) CCI produces cross-tolerance with morphine. These results indicate that common mechanisms may be involved in neuropathic pain and MTD. Modulation of PKC translocation and activation may prove useful for the treatment of pain and opiate addiction.

REFERENCES

Aldinio, C.; Valenti, G.; Savoini, G.E.; Kirschner, G.; Agnati, L.F.; and Toffano, G. Monosialoganglioside internal ester stimulates the dopaminergic reinnervation in rat. *Int J Dev Neurosci* 2:267-275, 1984.

Alho, H.; Favaron, M.; Putzolo, P.; Guidotti, A.; and Costa, E. Glutamate neurotoxicity and protective effects of gangliosides studied at the ultrastructural level in cultured neurons. *FASEB J* 2:A824, 1988.

Alkon, D.L., and Rasmussen, H. A spatial-temporal model of cell activation. *Science* 239:998-1005, 1988.

Allbritton, N.L.; Meyer, T.; and Stryer, L. Range of messenger action of calcium ion and inositol 1,4,5-trisphosphate. *Science* 258:1812-1815, 1992.

Attal, N.; Jazat, F.; Kayser, V.; and Guilbaud, G. Further evidence for 'pain-related' behaviours in a model of unilateral peripheral mononeuropathy. *Pain* 41:235-251, 1990.

Bassi, S.; Albizzati, M.G.; Sbacchi, M.; Frattola, L.; and Massarotti, M. Double-blind evaluation of monosialogaglioside (GM 1) therapy in stroke. *J Neurosci Res* 12:493-498, 1984.

Bennett, G.J., and Ochoa, J.L. Thermographic observations on rats with experimental neuropathic pain. *Pain* 45:61-67, 1991.

Bennett, G.J., and Xie, Y.K. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain* 33:87-107, 1988.

Bessou, P., and Perl, E.R. Response of cutaneous sensory units with unmyelinated fibers to noxious stimuli. *J Neurophysiol* 32:1025-1043, 1969.

Bianchi, R.; Marini, P.; Merlini, S.; Fabris, M.; Triban, C.; Mussini, E.; and Friori, M.G. ATPase activity defects in alloxan-induced diabetic sciatic nerve recovered by ganglioside treatment. *Diabetes* 37:1340-1345, 1988.

Bose, B.; Osterholm, J.L.; and Kalia, M. Ganglioside-induced regeneration and reestablishment of axonal continuity in spinal cord-transected rats. *Neurosci Lett* 63:165-169, 1986.

Caccia, M.R.; Meola, G.; Cerri, C.; Frattolo, L.; Scarlato, G.; and Aporti, F. Treatment of denervated muscle by gangliosides. *Muscle Nerve* 2:382-389, 1979.

Campbell, J.N.; Meyer, R.A.; and LaMotte, R.H. Sensitization of myelinated nociceptive afferents that innervate monkey hand. *J Neurophysiol* 42:1669-1679, 1979.

Chen, L., and Huang, L.Y. Sustained potentiation of NMDA receptor-mediated glutamate responses through activation of protein kinase C by a mu opioid. *Neuron* 7:319-326, 1991.

Chen, L., and Huang, L.Y. Protein kinase C reduces Mg2+ block of NMDA-receptor channels as a mechanism of modulation. *Nature* 356:521-523, 1992.

Choi, D.W. Cerebral hypoxia: Some new approaches and unanswered questions. *J Neurosci* 10:2493-2501, 1990.

Chou, D.K.H.; Dodd, J.; Jessell, T.M.; Costello, C.E.; and Jungalwala, F.B. Identification of alpha-gapactose (alpha-fucose)-asialoGM1 glycolipid expressed by subsets of rat dorsal root ganglion neurons. *J Biol Chem* 264:3409-3415, 1989.

Collin, E., and Cesselin, F. Neurobiological mechanisms of opioid tolerance and dependence. *Clin Neuropharmacol* 14:465-488, 1991.

Collingridge, G.L.; Randall, A.D.; Davies, C.H.; and Alford, S. The synaptic activation of NMDA receptors and Ca2+ signalling in neurons. *Ciba Found Symp* 164:162-171, 1992.

Collingridge, G.L., and Singer, W. Excitatory amino acid receptors and synaptic plasticity. *Trends Pharmacol Sci* 11:290-296, 1990.

Costa, E., and Rodbell, M. Receptor-mediated engram: Signal transduction and long-term modification of neuronal receptor responses. *Psychopharmacol Bull* 24:403-407, 1988.

Cotman, C., and Monaghan, D. Multiple excitatory amino acid receptor regulation of intracellular Ca: Implications for aging and Alzheimer's disease. *Ann N Y Acad Sci* 568:138-148, 1989.

D'Amour, F.E., and Smith, D.L. A method for determining loss of pain sensation. *J Pharmacol Exp Ther* 72:74-79, 1941.

Davar, G., and Maciewicz, R.J. Deafferentation pain syndromes. *Neurol Clin* 7:289-384, 1989.

De Montis, M.G.; Devoto, P.; Meloni, D.; Gambarana, C.; Giorgi, G.; and Tagliamonte, A. NMDA receptor inhibition prevents tolerance to cocaine. *Pharmacol Biochem Behav* 42:179-182, 1992.

Devor, M. Nerve pathophysiology and mechanisms of pain in causalgia. *J Auton New Syst* 7:371-384, 1983.

Di Gregorio, F.; Favaro, G.; Panozzo, C.; and Fiori, M.G. Efficacy of ganglioside treatment in reducing functional alterations induced by vincristine in rabbit peripheral nerves. *Cancer Chemother Pharmacol* 26:31-36, 1990.

Domanska Janik, K., and Zalewska, T. Effect of brain ischemia on protein kinase *C. J Neurochem* 58:1432-1439, 1992.

Dubner, R. Neuronal plasticity and pain following peripheral tissue inflammation or nerve injury. In: Bond, M.; Charlton, E.; and Woolf, C.J., eds. *Proceedings of Sixth World Congress on Pain. Pain Research and Clinical Management.* Vo1 5. Amsterdam: Elsevier, 1991. pp. 263-276.

Dunbar, G.L.; Butler, W.M.; Fass, B.; and Stein, D.G. Behavioral and neurochemical alterations induced by exogenous gangliosides in brain damaged animals: Problems and prospectives. In: Tettamanti, G.; Ledeen, R.W.; Sandhoff, K.; Nagai, Y.; and Toffano, G., eds. *Gangliosides and Neuronal Plasticity.* New York: Liviana Press, 1986. pp. 365-380.

Eccles, J.C. Calcium in long-term potentiation as a model for memory. *Neuroscience* 10:1071-1081, 1983.

Facci, L.; Leon, A.; and Skaper, S.D. Excitatory amino acid neurotoxicity in cultured retinal neurons: Involvement of N-methyl-D-aspartate (NMDA) and non-NMDA receptors and effect of ganglioside GM 1. *J Neurosci Res* 27:202-210, 1990*b*.

Facci, L.; Leon, A.; and Skaper, S.D. Hypoglycemic neurotoxicity in vitro: Involvement of excitatory amino acid receptors and attenuation by monosialoganglioside GM1 . *Neuroscience* 37:709-716, 1990*a* .

Favaro, G.; Di Gregorio, F.; Panozzo, C.; and Fiori, M.G. Ganglioside treatment of vincristine-induced neuropathy: An electrophysiologic study. *Toxicology* 49:325-329, 1988.

Favaron, M.; Manev, H.; Alho, H.; Bertolino, M.; Ferret, B.; Guidotti, A.; and Costa, E. Gangliosides prevent glutamate neurotoxicity in neuronal cultures. *Proc Natl Acad Sci U S A* 85:7351-7355, 1988.

Fox, K., and Daw, N.W. Do NMDA receptors have a critical function in visual cortical plasticity? *Trends Neurosci* 16:116-122, 1993.

Gorio, A.; Marini, P.; and Zanoni, R. Muscle reinnervation: III. Motoneuron sprouting capacity, enhancement by exogenous gangliosides. *Neuroscience* 8:417-429, 1983.

Govrin-Lippmann, R., and Devor, M. Ongoing activity in severed nerves: Source and variation with time. *Brain Res* 159:406-410, 1978.

Hayes, R.L.; Jenkins, L.W.; Lyeth, B.G.; Balster, R.L.; Robinson, S.E.; Clifton, G.L.; Stubbins, J.F.; and Young, H.F. Pretreatment with phencyclidine, an N-methyl-D-aspartate antagonist, attenuates long-term behavioral deficits in the rat produced by traumatic brain injury. *J Neurotrauma* 5:259-274, 1988.

Hayes, R.L.; Mao, J.; Price, D.D.; Germano, A.; dAvella, D.; Fiori, M.; and Mayer, D.J. Pretreatment with gangliosides reduces abnormal nociceptive responses associated with a rodent peripheral mononeuropathy. *Pain* 48:391-396, 1992.

Horowitz, S.H. Ganglioside therapy in diabetic neuropathy. *Muscle Nerve* 9:531-536, 1986.

Janig, W. Pathophysiology of nerve following mechanical injury. In: Dubner, R.; Gebhart, G.F.; and Bond, M.R., eds. *Proceedings of the Fifth World Congress on Pain.* New York: Elsevier, 1988. pp. 89-108.

Kaczmarek, L.K. The role of protein kinase C in the regulation of ion channels and neurotransmitter release. *Trends Neurosci* 10:30-34, 1987.

Karpiak, S.E.; Li, Y.S.; arid Mahadik, S.P. Gangliosides (GM1 and AGF2) reduce mortality due to ischemia: Protection of membrane function. *Stroke* 18:184-187, 1987*a.*

Karpiak, S.E.; Li, Y.S.; and Mahadik, S.P. Ganglioside treatment: Reduction of CNS injury and facilitation of functional recovery. *Brain Inj* 1:161-170, 19873.

Kojima, H.; Gorio, A.; Janigro, D.; and Jonsson, G. GM1 ganglioside enhances regrowth of noradrenaline nerve terminals in rat cerebral cortex lesioned by the neurotoxin 6-hydroxydopamine. *Neuroscience* 13:1011-1022, 1984.

Kupers, R.C.; Konings, H.; Adriaensen, H.; and Gybels, J.M. Morphine differentially affects the sensory and affective pain ratings in neurogenic and idiopathic forms of pain. *Pain* 47:5-12, 1991.

Laird, J.M.A., and Bennett, G.J. Responses of spinal dorsal horn neurons in rats with an experimental peripheral mononeuropathy. *Soc Neurosci Abstr* 17:1991.

Leon, A.; Lipartiti, M.; Seren, M.S.; Lazzaro, A.; Mazzari, S.; Koga, T.; Toffano, G.; and Skaper, SD. Hypoxic-ischemic damage and the neuroprotective effects of GM1 ganglioside. *Stroke* 21:95-97, 1990.

Li, Y.S.; Mahadik, S.P.; Rapport, M.M.; and Karpiak, S.E. Acute effects of GM1 ganglioside: Reduction in both behavioral asymmetry and loss of Na+, K+-ATPase after nigrostriatal transection. *Brain Res* 377:292-297, 1986.

Lindblom, U.L.F. Classification and assessment of altered sensation and pain. In: Dimitrijevic, M.R.; Wall, P.D.; and Lindblom, U.L.F., eds. *Recent Achievements in Restorative Neurology.* Vol. III, *Altered Sensation and Pain.* New York: Karger, 1990. pp. 7-16.

Linden, D.J.; Wong, K.L.; Sheu, F.S.; and Routtenberg, A. NMDA receptor blockade prevents the increase in protein kinase C substrate (protein F1) phosphorylation produced by long-term potentiation. *Brain Res* 458:142-146, 1988.

Lombardi, G.; Beni, M.; Consolazione, A.; and Moroni, F. Lesioning and recovery of the serotoninergic hippocampal afferents: Differential effects of GM1 ganglioside. *Neuropharmacology* 27:1085-1088, 1988.

Maciewicz, R.J., and Fields, H. Pain pathways. In: Asbury, A.K.; McKhann, G.M.; and McDonald, W.I., eds. *Diseases of the Nervous System.* Philadelphia: W.B. Saunders Company, 1986. pp. 930-941.

Madison, D.V.; Malenka, R.C.; and Nicoll, R.A. Mechanisms underlying long-term potentiation of synaptic transmission. *Annu Rev Neurosci* 14:379-397, 1991.

Magal, E.; Louis, J.C.; Aguilera, J.; and Yavin, E. Gangliosides prevent ischemia-induced down-regulation of protein kinase C in fetal rat brain. *J Neurochem* 55:2126-2131, 1990.

Mahadik, S.P.; Hawver, D.B.; Hungund, B.L.; Li, Y.S.; and Karpiak, S.E. GM1 ganglioside treatment after global ischemia protects changes in membrane fatty acids and properties of Na+, K+- ATPase and Mg2+-ATPase. *J Neurosci Res* 24:402-412, 1989.

Mao, J.; Hayes, R.L.; Price, D.D.; Coghill, R.C.; Lu, J.; and Mayer, D.J. Post-injury treatment with GM1 ganglioside reduces nociceptive behaviors and spinal cord metabolic activity in rats with experimental peripheral mononeuropathy. *Brain Res* 584:18-27, 1992*a*.

Mao, J.; Price, D.D.; Coghill, R.C.; Mayer, D.J.; and Hayes, R.L. Spatial patterns of spinal cord-2-deoxyglucose metabolic activity in a rat model of painful peripheral mononeuropathy. *Pain* 50:89-100, 1992*b*.

Mao, J.; Price, D.D.; Hayes, R.L.; Lu, J.; and Mayer, D.J. Differential roles of NMDA and non-NMDA receptor activation in induction and maintenance of thermal hyperalgesia in rats with painful peripheral mononeuropathy. *Brain Res* 598:271-278, 1992*d*.

Mao, J.; Price, D.D.; Hayes, R.L.; Lu, J.; and Mayer, D.J. Intrathecal GM 1 ganglioside and local nerve anesthesia reduce nociceptive behaviors in rats with experimental peripheral mononeuropathy. *Brain Res* 584:28-53, 1992*c*.

Mao, J.; Price, D.D.; Mayer, D.J.; and Hayes, R.L. Pain-related increases in spinal cord membrane-bound protein kinase C following peripheral nerve injury. *Brain Res* 588:144-149, 1992*e*.

Mao, J.; Price, D.D.; Mayer, D.J.; Lu, J.; and Hayes, R.L. Intrathecal MK-801 and local nerve anesthesia synergistically reduce nociceptive behaviors in rats with experimental peripheral mononeuropathy. *Brain Res* 576:254-262, 1992*f*.

Mao, J.; Mayer, D.J.; and Price, D.D. Patterns of increased brain activity indicative of pain in a rat model of peripheral mononeuropathy. *J Neurosci* 13:2689-2702, 1993.

Mao, J.; Price, D.D.; and Mayer, D.J. Thermal hyperalgesia in association with the development of morhine tolerance in rats: Roles of excitatory amino acids and protein kinase C. *J Neurosci* 14:2301-2312, 1994.

Mao, J.; Price, D.D.; and Mayer, D.J. Tolerance to morphine analgesia induced by an experimental painful mononeuropathy in rats. *Pain.* Manuscript submitted for publication.

Marek, P.; Ben Eliyahu, S.; Gold, M.; and Liebeskind, J.C. Excitatory amino acid antagonists (kynurenic acid and MK-801) attenuate the development of morphine tolerance in the rat. *Brain Res* 547:77-81, 1991.

Massarotti, M. Ganglioside therapy of peripheral neuropathies: A review of clinical literature. In: Tettamanti, G.; Ledeen, R.W.; Sandhoff, K.; Nagai, Y.; and Toffano, G., eds. *Gangliosides and Neuronal Plasticity.* New York: Liviana Press, 1986. pp. 465-479.

Mayer, M.L., and Miller, R.J. Excitatory amino acid receptors, second messengers and regulation of intracellular Ca2+ in mammalian neurons. *Trends Pharmacol Sci* 11:254-260, 1990.

Mayer, D.J.; Wolfle, T.L.; Akil, H.; Carder, B.; and Liebeskind, J.C. Analgesia from electrical stimulation in the brainstem of the rat. *Science* 174:1351-1354, 1971.

Merskey, H. Pain terms: A current list with definitions and notes on usage. Supplement. *Pain* 3:S215-S221, 1986.

Miyamoto, T., and Okada, Y. NMDA receptor, protein kinase C and calmodulin system participate in the long-term potentiation in guinea pig superior colliculus slices. *Brain Res* 605:287-292, 1993.

Nagai, Y., and Iwamori, M. Ganglioside distribution at different levels of organization and its biological implications. In: Ledeen, R.W.; Yu, R.K.; Rapport, M.M.; and Suzuki, K., eds. *Ganglioside Structure, Function, and Biological Potential.* New York: Plenum Press, 1984. pp. 135-146.

Nishizuka, Y. The family of protein kinase C for signal transduction. *JAMA* 262:1826-1833, 1989.

Numann, R.; Catterall, W.A.; and Scheuer, T. Functional modulation of brain sodium channels by protein kinase C phosphorylation. *Science* 254:115-118, 1991.

Olds, J.L.; Anderson, M.L.; McPhie, D.L.; Staten, L.D.; and Alkon, D.L. Imaging of memory-specific changes in the distribution of protein kinase C in the hippocampus. *Science* 245:866-869, 1989.

Palecek, J.; Paleckova, V.; Dougherty, P.M.; Carlton, S.M.; and Willis, W.D. Responses of spinothalamic tract cells to mechanical and thermal stimulation of skin in rats with experimental peripheral neuropathy. *J Neurophysiol* 67:1562-1573, 1992.

Portenoy, R.K.; Foley, K.M.; and Inturrisi, C.E. The nature of opioid responsiveness and its implications for neuropathic pain: New hypotheses derived from studies of opioid infusions. *Pain* 43:273-286, 1990.

Price, D.D. *Psychological and Neural Mechanisms of Pain.* New York: Raven Press, 1989.

Price, D.D.; Mao, J.R.; Coghill, R.C.; dAvella, D.; Cicciarello, R.; Fiori, M.G.; Mayer, D.J.; and Hayes, R.L. Regional changes in spinal cord glucose metabolism in a rat model of painful neuropathy. *Brain Res* 564:314-318, 1991.

Probst, W.; Mobius, D.; and Rahmann, H. Modulatory effects of different temperatures and Ca concentrations on gangliosides and phospholipids in monolayer air/water interfaces and their possible functional role. *Cell Mol Neurobiol* 4:157-176, 1984.

Rahmann, H. Functional implications of gangliosides in synaptic transmission. *Neurochem Int* 5:539-547, 1983.

Rodden, F.A.; Wiegandt, H.; and Bauer, B.L. Gangliosides: The relevance of current research to neurosurgery. *J Neurosurg* 74:606-619, 1991.

Sabel, B.A.; DelMastro, R.; Dunbar, G.L.; and Stein, D.G. Reduction of anterograde degeneration in brain damaged rats by GM1-gangliosides. *Neurosci Lett* 7:360-366, 1987.

Sabel, B.A., and Schneider, G.E. Enhanced sprouting of retinotectal fibers after early superior colliculus lesions in hamsters treated with gangliosides. *Exp Brain Res* 71:365-376, 1988.

Sato, J., and Perl, E.R. Adrenergic excitation of cutaneous pain receptors induced by peripheral nerve injury. *Science* 251:1608-1610, 1991.

Schengrund, C.L. The role(s) of gangliosides in neural differentiation and repair: A prospective. *Brain Res BUll* 24:131-141, 1990.

Schulman, H., and Lou, L.L. Multifunctional Ca2+/calmodulin-dependent protein kinase: Domain structure and regulation. *Trends Biochem Sci* 14:62-66, 1989.

Smimova, T.; Stinnakre, J.; and Mallet, J. Characterization of a presynaptic glutamate receptor. *Science* 262:430-433, 1993.

Spoerri, P.E. Facilitated-establishment of contacts and synapses in neuronal cultures: Ganglioside-mediated neurite sprouting and outgrowth. In: Tettamanti, G.; Ledeen, R.W.; Sandhoff, K.; Nagai, Y.; and Toffano, *G.,* eds. *Gangliosides and Neuronal Plasticity.* New York: Liviana Press, 1986. pp. 309-326.

Stewart, J.D., and Aguayo, A.J. Compression and entrapment neuropathies. In: Dyck, P.J.; Thomas, P.K.; Lambert, E.H.; and Bunge, R., eds. *Peripheral Neuropathy.* Philadelphia: W.B. Saunders Company, 1984. pp. 1435-1457.

Sugimoto, T.; Bennett, G.J.; and Kajander, K.C. Strychnine-enhanced transsynaptic degeneration of dorsal horn neurons in rats with an experimental painful peripheral neuropathy. *Neurosci Lett 98:* 139-143, 1989.

Sugimoto, T.; Bennett, G.J.; and Kajander, K.C. Transsynaptic degeneration in the superficial dorsal horn after sciatic nerve injury: Effects of a chronic constriction injury, transection, and strychnine. *Pain* 42:205-213, 1990.

Thomas, P.K. Clinical features and differential diagnosis of peripheral neuropathy. In: Dyck, P.J.; Thomas, P.K.; Lambert, E.H.; and Bunge, R., eds. *Peripheral Neuropathy.* Philadelphia: W.B . Saunders Company, 1984. pp. 1169-1190.

Tiseo, P.J., and Inturrisi, C.E. Attenuation and reversal of morphine tolerance by the competitive N-methyl-D-aspartate receptor antagonist, LY274614. *J Pharmacol Exp Ther* 264:1090-1096, 1993.

Triban, C.; Guidolin, D.; Fabris, M.; Marini, P.; Schiavinato, A.; Dona, M.; Bortolami, M.C.; Di Giamberardino, L.; and Fiori, M.G. Ganglioside treatment and improved axonal regeneration capacity in experimental diabetic neuropathy. *Diabetes* 38:1012-1022, 1989.

Trilivas, I.; McDonough, P.M.; and Brown, J.H. Dissociation of protein kinase C redistribution from the phosphorylation of its substrates. *J Biol Chem* 266:8431-8438, 1991.

Trujillo, K.A., and Akil, H. Inhibition of morphine tolerance and dependence by the NMDA receptor antagonist MK-801. *Science* 251:85-87, 1991*a*.

Trujillo, K.A., and Akil, H. Opioid tolerance and dependence: Recent findings and synthesis. New *Biol* 3:915-923, 1991*b*.

Vaccarino, F.; Guidotti, A.; and Costa, E. Ganglioside inhibition of glutamate-mediated protein kinase C translocation in primary cultures of cerebelar neurons. *Proc Natl Acad Sci U S A* 34:8707, 1987.

Wakisaka, S.; Kajander, K.C.; and Bennett, G.J. Abnormal skin temperature and abnormal sympathetic vasomotor innervation in an experimental painful peripheral neuropathy. *Pain* 46:299-313, 1991.

Wall, P.D. The prevention of postoperative pain. *Pain* 33:289-290, 1988.

Wall, P.D., and Gutnick, M. Ongoing activity in peripheral nerves. The physiology and pharmacology of impule originating from a neuroma. *Exp Neural* 43:580-593, 1974.

West, J.W.; Numann, R.; Murphy, B.J.; Scheuer, T.; and Catterall, W.A. A phosphorylation site in the Na+ channel required for modulation by protein kinase C. *Science* 254:866-868, 1991.

Wilcox, G. Excitatory neurotransmitters and pain. In: Bond, M.R.; Charlton, J.E.; and Woolf, C.J., eds. *Proceedings of the Sixth World Congress on Pain. New* York: Elsevier, 1991. pp. 97-114.

Willis, W.D. *The Pain System.* Basel: Karger, 1985.

Worley, P.F.; Baraban, J.M.; and Snyder, S.H. Heterogeneous localization of protein kinase C in rat brain: Autoradiographic analysis of phorbol ester receptor binding. *J Neurosci* 6:199-207, 1986.

Wu, P.H.; Mihic, S.J.; Liu, J.F.; Le, A.D.; and Kalant, H. Blockade of chronic tolerance to ethanol by the NMDA antagonist, (+)-MK-801. *Eur J Pharmacol* 231:157-164, 1993.

Yaksh, T.L. The effects of intrathecally administered opioid and adrenergic agents on spinal function. In: Yaksh, T.L., ed. *Spinal Afferent Processing.* New York: Plenum Press, 1986. pp. 505-539.

Yamamoto, T., and Yaksh, T.L. Spinal pharmacology of thermal hyperalgesia induced by constriction injury of sciatic nerve: Excitatory amino acid antagonists. *Pain* 49:121-128, 1992.

Yavin, E. Modulation of neurite outgrowth and ganglioside associated changes in cerebral neurons and PC12 pheochromocytoma cells. In: Tettamanti, G.; Ledeen, R.W.; Sandhoff, K.; Nagai, Y.; and Toffano, *G.,* eds. *Gangliosides and Neuronal Plasticity.* New York: Liviana Press, 1986. pp. 257-270.

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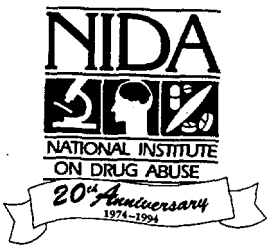

NIH Publication No. 95-3887
Printed 1995