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Neuroscience Methods in Drug Abuse Research

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Preface

The explosive growth in neuroscience research over the last decade
is paralleled by the impressive array of new approaches now being
applied in drug abuse research. In particular, techniques in
neuroanatomy, neurophysiology, and molecular biology are now coming
into prominence along with the more classical pharmacological and
biochemical techniques that once dominated the drug abuse field.
More importantly, however, it has been the creative use of
multidisciplinary tactics, as illustrated by the papers in this
review, that has substantially increased the impact of technological
advances in the drug abuse field. The papers in this monograph are
based on a technical review held on September 9 and 10, 1984, and
are presented in three sections. We hope these papers will
stimulate multidisciplinary research on drug abuse issues by serving
as examples, as well as information sources, to the field.

In the first section, the application of _in_ _vitro_ autoradiography to
brain and drug abuse research is described by Michael Kuhar and
Miles Herkenham. This method, which has supplied us with a high
resolution technique for localizing receptors to drugs and neuro-
transmitters in the central nervous system, is described and
analyzed. This analysis is timely now, because after an exciting
decade of use as a qualitative tool, autoradiography is about to
become even more productive as a quantitative technique as well.
The authors concentrate on the promises and problems associated with
quantitative autoradiography and supply some of the best background
material available on the subject.

In the second section, approaches integrating electrophyslology with
behavioral analysis are applied to the study of drug actions on the
nervous system. This section begins with a description of a unique
application of a now classical technique in drug abuse research,
electrical self-stimulation of the brain's reward system. These
studies, by Conan Kornetsky, use the subjects' willingness to work
for intracranial electrical stimulation to define what is and is not
rewarding and illustrate how a drug's interaction with this
behavioral model is a useful predictor of abuse liability. John
Chapin and and his colleagues and Sam Deadwyler show how to relate
neural activity to complex behaviors in experimental animals. In
their analyses, they show how drugs alter the activity of both
single neurons and ensembles of neurons, and that the drug-induced
changes in neural activity are correlated with and may predict overt
behavioral effects of drugs. Chapin uses a paradigm that explores
sensorimotor integration, while Deadwyler's model focuses on sensory
processing as it relates to higher cognitive functions. Finally,

Henri Begleiter and Bernice Porjesz describe the use of evoked brain potentials to study the effects of drugs on humans. Their work with alcoholics, using a computer-assisted analysis of the electrical brain activity associated with both simple and complex cognitive functions, has revealed alterations in CNS activity after acute and chronic alcohol exposure, after abstinence from alcohol, and even in the offspring of the alcoholics. These fascinating studies suggest that it may be possible to find markers for individuals who are susceptible to drug abuse. Clearly, electrophysiology is undergoing a renaissance in neuroscience research. This revival has been aided by the integration of the computer into the laboratory, but would not have progressed as it has without the creative drive of investigators such as those who have contributed to this section.

In the final section, some of the consequences of chronic drug use are explored. Lewis Seiden describes, by a variety of neuropharma-cological, neurochemical. and histological studies, the methampheta-mine-induced loss of dopamine and dopamine-containing neural elements in the striatum. This toxic effect may occur after a single high dose or a more prolonged, lower dose regimen. Interest-ingly, the damage caused by methamphetamine results from the brain's own dopamine. This startling finding suggests that neurotoxic consequences may follow the abuse of other drugs that act to block monoamine reuptake mechanisms. Horace Loh and his colleagues summarize their work on opiate mechanisms in which they have used a neuroblastoma x glioma hybrid cell line that contains delta opiate receptors. They have used this cell line as a model to study the molecular mechanisms of tolerance and dependence. In this model system, tolerance is manifested by the loss of the opiate-induced inhibition of adenylate cyclase upon prolonged opiate adminis-tration. This loss appears to be caused by an initial decrease in opiate receptor affinity followed by an actual loss of opiate receptors in the external membrane of the cell. Dependence is manifested by a naloxone-induced increase in adenylate cyclase activity after chronic opiate exposure. This is related not to changes in the opiate receptor, but to the accumulation of two cytosolic components: adenosine and (tentatively) calmodulin. These substances activate adenylate cyclase, although the relationship of this activation to naloxone occupation of opiate receptors is not understood. Finally, Sandra Moon describes the effects of _in utero_ drug exposure on developing neurochemical systems. This work, which utilizes both receptor autoradiography and fluorescence to reveal changes in opiate receptors and dopamine in the striatum caused by haloperidol administration, shows how alterations in one neuro-chemical system may alter other systems. It also illustrates the kind of creative use of anatomical techniques now occurring in drug abuse research.

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Contents

Receptor Mapping in Neuro-pharmacology by Autoradiography: Some Technical Problems

Michael J. Kuhar, James R. Unnerstall, and
Errol B. De Souza

INTRODUCTION

Techniques for receptor mapping have been steadily improving
for the past decade. Microscopic receptor mapping is now as
feasible and popular as neurotranamitter and enzyme
histochemiatry has been for many years. A main reason for this
has been the development and adaptation of microscopic
autoradiographic methods in parallel with the biochemical
binding techniques which have so dramatically advanced the
study of receptors at the molecular level. The goals of this
chapter are to summarize the main issues in receptor mapping by
autoradiography, to discuss some important current technical
problems which profoundly affect the methods that one should
select to do experiments, and also to discuss the
interpretation of autoradiograma.

The fundamental advantages of microscopic receptor mapping are
increased anatomical resolution and greatly increased
sensitivity of measurement (at least when compared to
biochemical approaches). The increase in anatomical resolution
is obvious because these are microacropic techniques which
always have greater resolution than microdissection techniques.
The great sensitivity in measurement derives from the fact that
one can measure receptor densities in very small microscopic
regions. This increase in sensitivity can be orders of
magnitude greater than that found in biochemical studies.
Thus, in general, receptor mapping will be useful in any
situation requiring receptor measurement in small regions or in
situations where overall quantities (but not necessarily
densities) of receptors are low. An example of such a
situation is an experiment on the axonal flow of receptors;
while it can be done biochemically, light microscopic
autoradiography provides a sensitive method of measurement and
anatomical resolution not available by other means (Kuhar and
Zarbin 1984; Zarbin et al. 1983). Additional information on
other more general uses of receptor mapping is presented below.

1

In general, there are two important principles that one must keep in mind when carrying out receptor mapping experiments. In our experience, these principles are critical in guiding the investigator to carrying out a useful experiment. The first principle is selective or preferential labeling of receptors. This has been facilitated by the development of receptor binding techniques over the past decade. The development of large numbers of radioactive liganda and the availability of extensive pharmacological studies on many receptors has provided the ability to define quite precisely the type of receptor to which one is binding. A second principle is the selection of appropriate methods of visualization. This is important because one does not visualize the receptor directly, but rather the ligand which binds to it. Thus, one must produce an autoradiogram or image in such a way that one prevents or minimizes the diffusion of ligand away from the receptor. It does no good or is even misleading if a receptor is appropriately labeled but the ligand is lost from the tissue during a preparation process that obtains the image by any means. This is obviously not an important issue where irreversibly binding ligands are used. However, the vast majority of available liganda are reversible binding agents and the autoradiography must be done in a way which takes into account the diffusible nature of the signal (Stumpf and Roth 1966; Roth and Stumpf 1969; Young and Kuhar 1979; Barnard 1979). Perhaps a third principle should be added, i.e., a principle of appropriate interpretation. As will be discussed below, the use of tritium in producing autoradiograma has some special pitfalls because of the low energy of the beta particles emitted by tritium. The efficiency with which beta rays are converted into autoradiographic grains varies from region to region because of this low energy which results in a differential absorption of beta rays by different tissue regions. The absorption varies depending on the dry mass content of the region. This is discussed in more detail below.

There are two main experimental approaches to receptor mapping experiments. They are in _vitro_ labeling procedures and in _vivo_ labeling procedures. The first is the more popular and more versatile one. In in _vitro_ labeling procedures, slide-mounted tissue sections are incubated with radioactive ligands so that the receptors are labeled under very controlled conditions. Following the labeling, the slide-mounted tissue sections can be rinsed to remove nonreceptor-bound drug and improve specific to nonspecific binding ratios. In in _vivo_ labeling procedures, the receptors are labeled in intact tissues in _vivo_ after systemic administration of the drug (ligand). If the ligand has a very high affinity for the receptor, tracer quantities of the drug can be injected into the animal, and after a relatively short time interval, the drug is carried to the brain by the blood, diffuses into the brain, and binds to the receptors. The nonreceptorbound drug is then removed from the brain and other tissues by various excretory processes. The high affinity of the drug for the receptor causes a retention of the drug on or in the vicinity of the receptor molecule.

Detailed strategies for carrying out both in vivo and in vitro labeling experiments have been described (Kuhar 1982a, 1982b. 1983). In vitro labeling procedures have-many advantages over in vivo labeling procedures. Since the experiments are carried out in vitro, one can control the labeling conditions so that there is greater specificity and efficiency in the labeling. One can add inhibitors to prevent metabolism of the labeling ligand and one can also use ligands which would not cross the blood-brain barrier in in vivo labeling procedures. One can also quite easily compare various receptor distributions because it is possible to use different ligands with consecutive sections of tissue. Also, this approach has a relatively low cost since an entire animal does not have to be labeled with drug and it is possible to carry out studies with human postmortem tissues. Yet, an additional advantage of in vitro labeling is that one can quite precisely define the receptor type that one is studying because of parallel biochemical experiments that can be carried out with the slide-mounted tissue sections. Nevertheless, in vivo labeling experiments are still quite important. Recently, PET scanning images of receptors have been utilize because of the ability to label neurotransmitter receptors in vivo. These PET scanning procedures are noninvasive and provide an extraordinary opportunity to examine receptors in clinical populations (Wagner et al. 1983; Eckelman et al. 1984).

The Uses of Receptor Mapping

Historically at least, the study of receptors has been within the discipline of pharmacology. Perhaps this is the reason that receptor mapping has most often been considered a pharmacological tool. Receptor maps provide powerful insights into the mechanism of drug action by identifying those brain regions which have the receptors and, therefore, those areas which are affected by drug administration. There are many examples of this. One of the first applications of this approach may have involved the opiate drugs. Administration of opiate drugs causes a variety of physiological affects, including analgesia, pupillary constriction, respiratory depression, and suppression of various visceral reflexes. Perhaps the only way this wide range of physiological action can be fully understood is to associate the various physiological effects with receptors in various neuronal circuits which mediate the effects (Kuhar 1978, 1981). In other words, identifying the neuronal circuits which have the receptors and which will be affected by drug administration may provide an understanding of how the drug produces its effect. Moreover, receptor mapping studies can be an impetus for developing new drugs. For example, several studies have indicated that there are high densities of neurotensin receptors on dopamine neurons in the midbrain. Recent studies by Uhl and Kuhar (1984) show that these neurotensin receptors up regulate after chronic administration of antipsychotic drugs, indicating the involvement of

neurotensin in the nigrostriatal system and possibly in systems involved in schizophrenia. These studies suggest that drugs aimed at the neurotensin receptor might be useful in treating movement disorders or emotional disorders associated with dopaminergic systems.

Receptor mapping is also a valuable compliment to neurotransmitter mapping. Receptor maps help complete our view of the biochemical organization of the brain. Recent studies on the corticotropin-releasing factor (CRF) receptors (De Souza et al. 1984) have shown that these receptors are found in many regions of the brain where endogenous CRF-containing neurons have been identified. A part of the brain containing very high levels of CRF has been the median eminence, and this region also has very high levels of CRF receptors (De Souza et al. 1984). In addition, CRF receptors were found in many other areas of the brain indicating that CRF may be a neurotransmitter in the brain as well as a hypothalamic-releasing factor.

Other uses of receptor maps include identifying changes in neuropathology and also providing needed data for a variety of other types of experiments, such as locating targets for experiments involving direct drug injections.

TECHNICAL PROBLEMS

Problems With Diffusion

As has been discussed briefly above, diffusion of ligand in receptor mapping procedures can distort the image such that it provides wrong or misleading information. Thus, the development of methods for dealing with diffusible molecules by Lloyd Roth and Walter Stumpf has been very helpful (Stumpf and Roth 1966; Roth and Stumpf 1969). Several approaches have been developed to prevent diffusion of radioactive ligands. Perhaps the safest and most widely used are the "dry" techniques. In these procedures, the radiolabeled tissues are kept dry and not exposed to any solvents which might provide an opportunity for diffusion or leeching of drug until after the autoradiographic image is formed. Other approaches involve barriers, such as plastic or carbon films placed over the radiolabeled tissues prior to dipping in warm emulsion. The barrier would at least theoretically prevent the wet emulsion from promoting diffusion while still allowing the close apposition of emulsion for the creation of the image.

Young and Kuhar (1979) developed a procedure for labeling receptors in slide-mounted tissue sections. Autoradiograms were generated by apposing dry emulsion on flexible coverslips. Diffusion was minimized at every step in this procedure by keeping samples cold and dry whenever possible.

Yet another approach involve? fixation of ligands into tissues. Irreversibly binding ligands can be said to be "fixed." Another way to fix radioactive ligands in tissue sites is to expose the radiolabeled tissues to fixatives. Fixation by formaldehyde vapors has been used and is obviously advantageous to fixation with liquids where diffusion may occur before fixation. Herkenham and Pert (1982) have proposed the use of a method which includes the vapor fixation of radioactive ligands in slide-mounted tissue sections followed by wet defatting procedures prior to the dipping into emulsion for production of autoradiograms. Recently, it has been found that the defatting procedures subsequent to the vapor fixation are especially useful in that they eliminate the different absorption of tritium-emitted B particles that are found in gray and white matter. This issue is discussed in more detail below. Autoradiograms prepared by these procedures appear to be quite
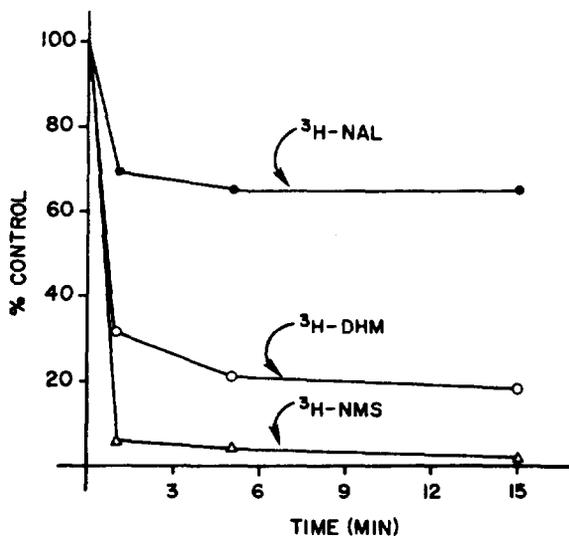
FIGURE 1

Time Course of [^{3}H]Ligand Loss in Ethanol Solution

Slide-mounted tissue sections were prepared and labeled with [^{3}H]dihydromorphine (DHM), [^{3}H]naloxone (NAL), or [^{3}H]N-methylscopolamine (NMS). Some slides were not incubated (controls) and others were incubated for varying amounts of time in 70% ethanol. Specific binding was then measured. See text for discussion. From Kuhar and Unnerstall (1982). Copyright 1982, Elsevier Scientific Publishers, B.V.

similar to those prepared by "dry" techniques where opportunity for diffusion is minimal. However, Kuhar and Unnerstall (1982) have found that the labeling ligand was at least partially lost from the tissue during the ethanol dehydration and xylene defatting treatment. At least one ligand, tritiated N-methylscopolamine (^{3}H-NMS), was totally lost from the tissue (figure 1). These results indicate that the vapor fixation by formaldehyde does not result in complete attachment of radioactive ligand to tissue elements. Also, the degree of attachment appears to vary with the ligand employed. Although it has not been shown, it is possible that the degree of attachment may vary also with the region of brain examined or possibly even with the subtype of receptor that the ligand is binding to. Thus, "diffusion" might not be the best description of the problem in this case, since the ligand which is fixed by the formaldehyde will not diffuse. However, "leeching" of ligand from tissue is a problem with this approach because not all of the ligand is covalently linked and there appears to be significant loss of ligand from the tissue during the wet dehydrating and defatting procedures. Stumpf and Roth (1966) examined the effect of gaseous formaldehyde fixation on steroid receptors labeling with ^{3}H-estradiol. These authors found that some degree of diffusion and loss of ligand from tissue definitely occurred, and they stated that one should not assume that fixation of tissue includes immobilization of the labeled compound. Studies by Kuhar and Unnerstall (1982) indicate that only partial fixation occurs for most ligand and, in some cases, at least for ^{3}H-NMS, no fixation of ligand occurs.

Thus, the introduction of dehydration, defatting, and wet emulsion application is not without problems, even with fixed tissues. The loss of label found by Kuhar and Unnerstall varies with the ligand chosen and opens the possibility that leeching of ligand will occur in the section to the extent that anatomical resolution will be reduced. Even if this were not a difficulty, the loss of label would require that proportionately longer exposure times be used. Also, the practically total loss of at least one ligand, 3H-NMS, indicates that these procedures are not generally applicable for receptor autoradiography. Further, while it has not been shown, it is important to rule out the possibility that preferential leeching will occur from some brain regions or from subtypes of receptors so that the resultant image may mislead investigators as to receptor distributions. Defatting does seem to have the significant advantage with tritium in that it reduces or eliminates the grey/white differences in efficiency described below (Herkenham and Sokoloff 1984).

Gray/White Differences with Tritium

Autoradiographic studies with tritium-labeled substances in brain have revealed differences in autoradiographic efficiency between gray and white matter (Alexander et al. 1981; Herkenham and Sokoloff 1984; Taylor et al. 1984). Basically, the finding

is that tritium in white matter did not produce
autoradiographic grains as efficiently as trituim in gray
matter (figure 2).
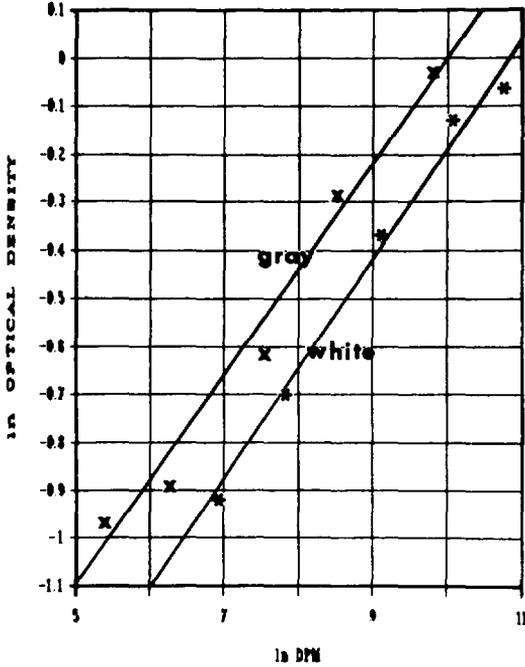
FIGURE 2

Double in Plots of Standard Data Indicating Different Auto-
radiographic Efficiency for Tritium in Grey and White Matter

Tissue sections from bovine caudate (grey) and internal capsule
(white) were incubated in varying concentrations of tritiated
isoleucine. DPM/section are not corrected for the dry mass of
the tissue. Optical densities were determined by a video-based
image analysis system. Further details are presented in the
text. Reproduced from J.R. Unnerstall's Ph.D. dissertation.

The problem appears to be that white matter has a higher dry
mass content than gray matter and, therefore, absorbs more beta
rays than does the gray matter. Significantly, it has been
found that the more energetic emissions from carbon-14 and
iodine-125 (I^{125}) do not show these gray/white differences.

These regional differences in efficiency with tritium are troublesome, particularly since most available ligands for receptor mapping are tritium labeled. If one attempts to quantify the receptor content in autoradiographic experiments, one must understand the relative "quenching" caused by different regions. Moreover, this is a difficult problem because the relative content of gray and white matter varies throughout the brain. Hence, investigators have sought some way to cope with these regional differences in efficiency. Kuhar, Unnerstall, and coworkers have attempted to develop a method for determining the relative amount of quenching in each brain region. These methods involve soaking slide-mounted tissue sections in tritium-labeled substances such that equal quantities of tritium are found in all regions of the tissue section. Autoradiograms produced from these sections showed varying grain densities and shoved the expected gray/white differences. Since the quantity of radioactivity in all brain regions was known, the relative efficiency of or a "quenching factor" for each brain region can be calculated. Another approach utilized by Kuhar and coworkers involved thaw mounting thin sections of brain on slides coated with tritium and other radioactive substances. As the beta rays passed through the thin sections of brain to form an image on film, the absorption of beta rays was proportional to the dry mass in the various regions. The image produced in these experiments also provided a means of directly calculating the relative quenching of a given brain region.

While these approaches have provided a way to cope with these differences in efficiency, they are obviously tedious and must be applied to every brain region studied. Also, it is possible that various experimental treatments will cause a difference in the tissue density of various brain regions and, hence, these techniques must be carried out for experimental animals as well as for controls.

Herkenham and Sokoloff (1984) found that defatting of slide-mounted tissue sections following formaldehyde vapor fixation resulted in a reduction of the gray/white matter difference in efficiency. It appears that the defatting procedures remove significant matter from the tissue sections so as to make the relative amount of quenching from gray and white matter regions the same. Thus, there is no need to correct optical densities for regional differences in efficiency. While these conclusions have not been supported with quantitative data from several brain regions, the qualitative information shown in autoradiograms from Herkenham and coworkers supports this idea quite clearly. Thus, defatting of the tissues after vapor fixation provides a substantial advantage in quantftation of autoradiograms with

tritium. However, as pointed out above, defatting procedures result in a significant loss of ligand from the tissues. Also, as stated above, this loss indicates that proportionately longer exposure times will be needed and also places one in danger that significant other artifacts may be occurring, as described above. Thus, it appears that neither approach with tritium is completely satisfactory.

Because of these problems, autoradiography with tritium can be undertaken only if attempts are made to understand the relative problems involved. Perhaps, the best solution will be to use I^{125}-labeled ligands. Not only do I^{125}-labeled ligands not have a regional efficiency problem, the specific activity of these ligands is substantially higher than those with tritium and much shorter autoradiographic exposure times are required.

Qusntitation of Autoradiograms

Aside from the above problem, the quantitation of autoradiograms is still not a trivial issue because the response of emulsions to radioactivity is not linear. In other words, increasing the radioactivity content of a tissue does not result in a linearly related increase in grain density or optical density. Several laboratories have explored this under conditions used for receptor mapping (Geary and Wooten 1983; Penney et al. 1981; Rainbow et al. 1982; Unnerstall et al. 1981, 1982) and found that appropriate translation of grain densities into femtomoles of ligand bound requires the use of standards to calibrate the experimental system used. While this is a difficult problem, it can be solved with a reasonable effort.

S U M M A R Y

In the past decade, substantial advances in receptor mapping have been made. It is now as feasible to carry out receptor localizations as it is to carry out neurotransmitter localizations. A great deal of data has accumulated on various technical procedures and it is clear that significant problems occur with tritiated ligands. These problems involve different autoradiographic efficiencies in tissue regions, and steps must be taken to either correct for these different efficiencies or to eliminate them. It seems that I^{125}-labeled ligands will become more and more important in the field.

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Levels of Quantitative Analysis of Receptor Autoradiography: Technical and Theoretical Issues

Miles Herkenham

Autoradiography is a tool used to localize radiolabeled substances contained within slide-mounted tissue sections. Labeled sections are apposed to a radiation-sensitive emulsion. When developed like a photograph, the exposed emulsion provides qualitative information about location of labeled material in different areas of a heterogeneous structure such as the brain. While anatomical localization can be highly informative (Pert and Herkenham 1981), often additional information about the amount of material incorporated is useful or necessary.

The autoradiographic film image results from the interaction of radiation (β particles) with silver halide crystals in the emulsion layer of the film, producing clusters of reduced silver atoms (Rogers 1979). The size of the clusters (grains) is a feature of the emulsion that affects the resolution and sensitivity of the film. The number of these grains produced is proportional to the level of radiation exposure. When developed, the silver grains are opaque, and the visible image is the result of different levels of light transmittance in different portions of the film. Quantifying the relative differences between different transmittance levels by densitometry seems to be a relatively easy matter, but assigning "absolute" values is considerably more difficult since, for example, the time of exposure would be a critical variable. In practice, the use of an independent measuring device, e.g., a liquid scintillation spectrofluorimeter, provides a means of assessing baseline values. However, for accurate assessment of radioactivity levels by either relative or absolute means, the response characteristics of the film over the range of radiation exposure must be known. The quantitative densitcmetric analysis of autoradiographs can be carried out at four distinct levels which are described Bela.

LEVELS OF QUANTITATIVE ANALYSIS

Rank Order of Density

Regardless of the amount of exposure (radioactive disintegrations x time), film transmittance levels can be assigned a rank order

which would be invariant for a given image. With autoradiography
of brain tissue sections containing receptor-bound radiolabeled
ligands, differential light transmittance through different parts
of the film will reflect differential amounts of ligand binding
sites across brain structures. Even this most basic form of quan-
tification is subject to several qualifications. First, since tri-
tium emits only weak particles, these emissions are differentially
self-absorbed ("quenched") in brain regions depending on the myelin
content in each region (Alexander et al. 1981; Herkenham and
Sokoloff 1984). As a result of differential quenching, two struc-
tures containing the same amount of radioactivity but different
amounts of white matter will show different transmittance levels
 (figure 1). Removing the lipids in the myelin by the histological
procedure of defatting after aldehyde fixation (Herkenham and Pert
1982) eliminates the differential quenching and "corrects" the
autoradiograph (Herkenham and Sokoloff 1984).

A second factor is that rank ordering is reliable only within the
same film image and, hence, only among areas in the same brain sec-
tion or among several sections exposed identically to the same film.
If rank ordering of structures contained in different brain sections
is required, the sections must also have been treated identically
 (identically prepared, co-incubated in vitro, washed and dried to-
gether).

Comparing the transmittance levels in different parts of the film is
usually accomplished by densitanetric analysis using some form of
photocell. The transmittance is translated to a measure of optical
density (OD), which will be discussed in greater detail below.

Magnitude of Relative Differences

 In order to describe "how much" different the radioactivity levels
are in different structures, or in the same structure after various
manipulations, assignment of numerical values to levels of film
darkness is needed. At this level of quantification, the absolute
magnitude of each value is not known, but relative differences among
structures can be expressed as a ratio, a proportion, or a percent
difference.   ODs are used in this way when the relationship between
OD and ligand bound has not been determined.

A densitometric analysis serves to quantify relative differences in
OD, but OD differences will not accurately reflect differences in li-
gand bound if the film response to radioactivity is not linear over
the range of values measured. For example, densitometry of two
structures with different amounts of radioactivity will show one
value of OD differences at a brief exposure and a smaller value at
a longer exposure if the film saturates to produce a "ceiling ef-
fect." The prudent investigator must determine the range over which
the film response is linear for the exposre levels encountered
and/or convert densitmtric data to values of radioactivity by the
use of standards prepared with known amounts of radioactivity and
tissue content. The remaininy parayraphs of this section describe
the theoretical and practical issues pertinant to the relationship
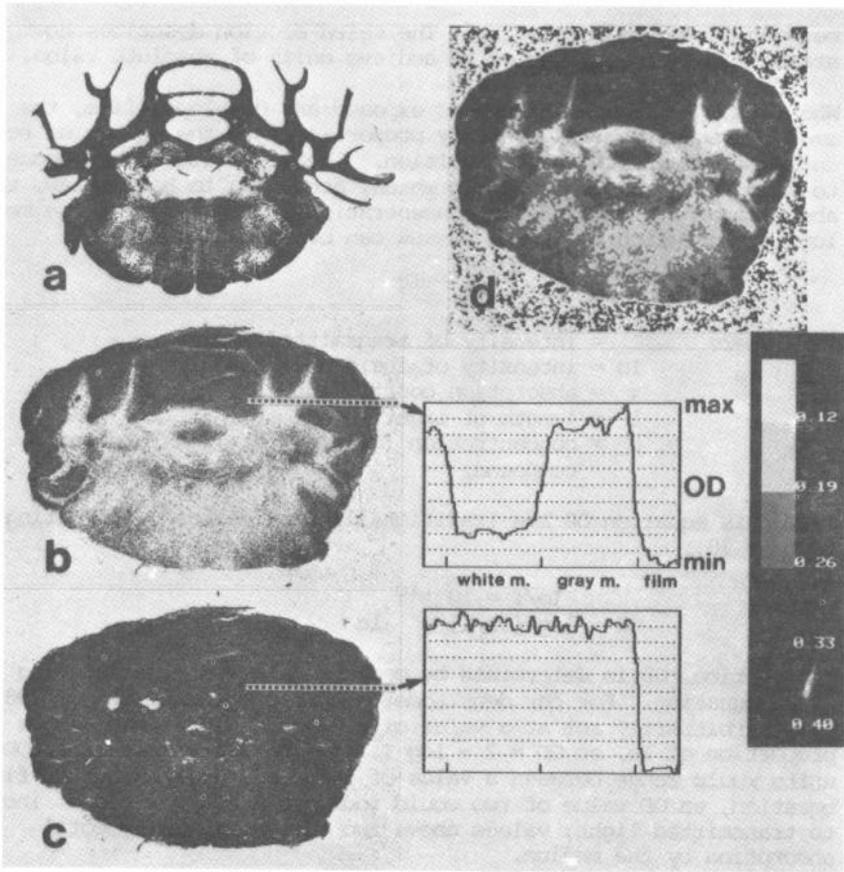
FIGURE 1

Regional Tritium Quenching

Silver stain of myelin (a) and autoradiographs of [³H]succinimidyl propionate binding (b-d) at level of rat cerebellum. Succinimidyl propionate is a protein acylating agent that binds homogeneously and covalently to all brain regions. The autoradiogaphs are all of the same section exposed before defatting (b and d) and again after defatting (c), both times for 10 days. The actual film images are photographed in b and c; d shows a computer-generated image in which ODs are coded in five shades of gray (as in the scale bar). The dotted lines in b and c show where computer-averaged densities were plotted as a function of horizontal position in a narrow rectangle containing cerebellar gray and white matter and the film. The histograms show relative ODs in these regions before and after defatting. From Herkenham and Sokoloff (1984).

between OD and radioactivity. The third section describes how
standards can be calibrated to achieve units of absolute value.

When light is passed through an exposed and developed film, the
amount transmitted is inversely proportional to the number of re-
duced silver grains in the emulsion. The situation is analogous
to absorption spectrometry, in which, according to Beer's Law, the
absorption of light and the concentration (or density) of the med-
ium through which the light passes can be expressed as:

$$I = Io \, e^{-\alpha lc}$$

where   I = intensity of transmitted light
         Io = intensity of incident light
         α = absorption coefficient
         1 = length of light path
         C = concentration (density) of light absorbing
              compound.

From this equation OD has traditionally been defined (converting
to base 10).

$$Io/I = 10^{\alpha lc}$$
$$OD = \log (Io/I) = \alpha lc$$

In practice, OD is determined by measuring Io and I and solving the
above equation. For our densitometry system (Goochee et al. 1980),
Io is arbitrarily set at a value of 100, and I is determined as a
proportion of Io, so OD = 2 - log I. In the latter instance, OD
units would range between a value of zero and two. Using the first
equation, an OD value of two would indicate a 100/1 ratio of incident
to transmitted light; values above two represent nearly total
absorption by the medium.

Since a and 1 are constants, OD should be <u>linearly</u> related to c,
the concentration of radioactivity (in units of exposure), but <u>only</u>
if the film <u>response</u> is linear. A plot of OD versus exposure should
reveal the response characteristics of the film.

Quantitative densitometry of receptors has thus far been done with
only one kind of film: a tritium-sensitive film called ^{3}H-Ultrofilm
(LKB, Gaithersburg, MD). However, despite its wide use for both
3H and [125]I I, the response characteristics of this film are not gen-
erally appreciated. A prerequisite for determining the response
characteristics has been the preparation of tritium standards. Sev-
eral method papers describing standards preparation and their appli-
cation to film autoradiography have been published (Penney et al.
1981; Unnerstall et al. 1982; Altar et al. 1984; Rainbow et al. 1984;
Geary et al., in press), but only a few have shown a plot of OD ver-
sus exposure. In one study such a plot revealed a curve best fit by
a logarithmic equation (Rainbow et al. 1984), while in another it
was fit by a rectangular hyperbola (Unnerstall et al. 1982). Using
a drum scanner system (Goochee et al. 1980), the plot of OD versus
radioactivity concentration in a set of commercially made standards

(tritium in plastic, from American Radiochemicals Corporation,
St. Louis, MO) resembles both these functions, but a power function
provides a closer fit (figure 2). Figure 2 shows that a linear
relationship between OD and expsure does not exist and is approxi-
mated only at very low OD values (Bela about 0.6). That is, LKB
film begins to saturate even with low exposures to tritium; complete
saturation occurs at or around an OD value of two.

The equation shown on the graph:

$$Y = aX^b$$

$$a = 0.148$$
$$b = 0.478$$
$$r^2 = 0.993$$
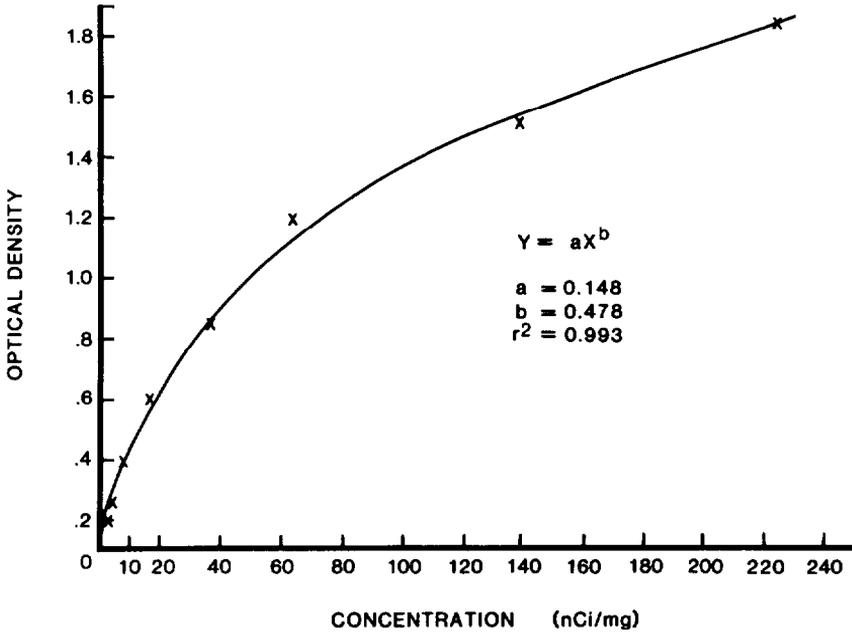
FIGURE 2

LKB Film Response

Eight different concentrations of tritium in plastic (commercial
standards) were apposed to film for 1 month, and their ODs were
measured as described. The relationship that best fits the result-
ing curve was computer derived. The deviation from linearity
represents the saturation of the film.

The LKB film response to radiation seems to be independent of the
nature of the source, as the saturation curve for 125I is very sim-
ilar to that for tritium (McLean et al., in press). This indicates
that saturation is a property of the film itself. The large grain
size of LKB film must play a major role in the shape of the satura-
tion curve. Of course, the higher energy 125I emissions expose the
film in hours or days instead of weeks or months for tritium.

When standards are used to convert ODs to units of radioactivity,
some aspects of film response to radiation should be considered. One

is that the rates of image generation are different for different
isotopes; because of this noncovariance, the isotope in the stan-
dard should be the same as in the tissue being masured (Geary et al.,
in press). Another consideration arises when comparing ODs of images
generated by the same isotope in two different media, such as plastic
and brain tissue. Though there is covariance of ^{3}H-tissue and ^{3}H-
plastic sources (Geary et al., in press), plastic quenches tritium
muchg more than does brain (Alexander et al. 1981). Therefore, equiv-
alent amounts of radioactivity in each would produce different ODs.
The values, therefore, can only be used in a relative fashion.

By the equation that describes the relationship between OD and tri-
tium exposure, a conversion from OD to units of radioactivity can be
made, permitting valid comparisons of relative differences in radio-
activity levels in different brain regions. Similarly, relative
differences in tritium quenching among different brain regions can
be quantified. For example, the quenching shwn in figure 1 was
quantified in table I of Herkenham and Sokoloff (1984). Cerebellar
gray and white matter had OD values of .35 and .09, respectively.
Using a power function that relates OD to radioactivity in plastic
at 2 weeks exposure, these values become 27.8 and 5.1 nCi/mg, re-
spectively. Thus, a 4-fold OD difference converts to a 5.4-fold
quench difference. In general, because of film saturation, OD dif-
ferences underestimate the magnitude of radioactivity differences;
furthermore, the underestimate increases with increasing ODs (as
emphasized by the changing slope in figure 2). Thus, conversion to
units of radioactivity is required for determining the relative dif-
ferences at any level of density.

In many studies the nonlinear response of the film is "corrected" by
logarithmic or log-log transformations. Traditionally, in photogra-
phy, OD has been plotted as a function of the logarithm of exposure
(Farnell 1966), producing the "H and D" curve, named after Hurter
and Driffield who defined OD in 1890 (Rogers 1979). The H and D
curve is S-shaped, with a long central straight portion (e.g., figure
2b of Unnerstall et al. 1982). This curve is instructive for several
reasons. First, since our eyes transduce light in a logarithmic
fashion, the log relationship approximates our perception of relative
"darkness." Second, the flattening at the low end is said to be an
index of film background, or "fog," whereas the high end shows some
practical limits of film saturation. Finally, the slope of the linear
portion is a measure of film "contrast" as we perceive it.

A linear plot of OD versus log exposure would be expected if the re-
lationship between OD and exposure is a logarithmic one. Hwever, in
our hands a pwer function was a better fit, so a log-log transforma-
tion should reveal a straight line. Figure 3 shws that this is the
case. The flattening at the low end seen with the log transformation
is nearly eliminated by the log-log transformation. This flattening
is more pronounced at low exposures (i.e., the same standards for one
week, not shown) but disapears if fog (OD of the film itself) is
subtracted. The flattening at the high end, for our densitometry
system at least, is mainly a consequence of our definition of OD as
2 - log I.
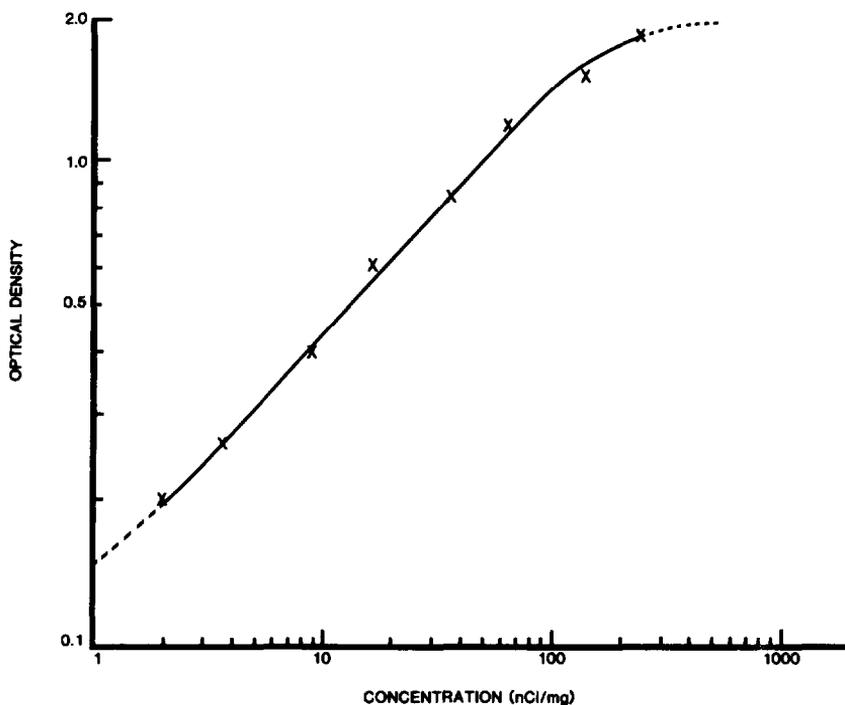
FIGURE 3

Log-Log Transformation of Film Response Data

The same data used for figure 2 are plotted on a log-log scale
here. A straight line best fits the data points. The deviations
from linearity at high OD values result from an OD ceiling of 2.0
and at low values from the contribution of film fog, or background,
which typically has an OD of 0.05 to 0.08.

Straight lines are often convenient for transforming and comparing
data. Hwever, logarithmically transformed data pose difficulties
for statistical analysis, since the magnitude of error of estimated
concentration, interpolated from the OD measurement, will be constant
only over the same range for which OD is linearly related to exposure.
As the slope of that relationship changes (figure 2), the error size
of estimated concentration increases with increasing ODs. Because
[3]H-Ultrofilm shows saturation over virtually the entire OD range,
statistics based on the assumption of constant error will be invalid.
Although   there   are   statistical   procedures   for   varying
error size (weighted linear regression, Snedecor and Cochran 1980),
for simplicity, a constant error might be approximated for OD values
on the "linear" part of the OD versus exposure curve, i.e., for OD
values below 0.6 (figure 2).

These issues are raised here not only because they affect how densi-
tometry data are analyzed, but because previous descriptions of LKB
film response to standards have used logarithmic data transformations
to empasize the "usable" or "workable" part of the OD range. Al-
though the "workable" range of film response might be considered as
that for which OD is linearly related to exposure, or that for which
a curve can be closely fit to relate the two, in other studies the
workable range is defined as that for which a straight line is found
after logarithmic (Unnerstall et al. 1982; both transformations in
figure 3 of Raibow et al. 1984) or log-log transformations (Geary
et al., in press). Since the data for the plastic standards are close-
ly fit with the densitometry system by a power function (figure 3),
the log-log transformation is linear over nearly the entire range of
the film. This result is much different than reported by others
(Unnerstall et al. 1982; Geary et al., in press), who saw deviation
fran linearity at OD values above 1.0 (the OD values reported by
Unnerstall et al. are x 100). Interestingly, in both studies
the reported deviation at ODs above 1.0 was based on only one data
point. Some studies also describe deviations at very lcw values of
OD, where signal detection becomes a major problem.

Thus, deviations from linearity can reflect several variables, includ-
ing errors in determining both ODs and the radioactivity in the stan-
dards, as well as a failure of the equation to closely fit the data.
Since film is not an "ideal" medium, we should not expect its response
to fit same "ideal" equation. In practice, whatever equation that most
closely describes the relationship between OD and exposure in the
standards is preferred--this may be one of the equations already de-
scribed, or it might be another, such as a polynomial (Reivich et al.
1969). When a cubic equation is used, for example, straight lines
after log or log-log transformations would not necessarily be expected.

In conclusion, LKB film response to tritium exposure is nonlinear.
Film saturation is noticeable at the lowest OD values, and so conver-
sion of OD units to units of radioactivity is necessary before com-
parisons of relative differences are valid. Conversion is achieved
by curve-fitting procedures: much of the OD range can be described
by a power function, but different equations may better fit narrow
intervals, especially at both low and high ends of the OD range.
Nearly all of the OD range is usable for quantification of relative
differences in radioactivity levels, whose accuracy is determined by
the closeness of fit provided by the equation that relates OD to ex-
posure and by the quality of the standards and the densitometer.
However, very little of the OD range can be used for statistics based
on the assumption of constant error size of estimated radioactivity
over the range of measured OD values. A log or log-log transforma-
ation of the data obscures the fact of changing error size, but it is
of value for comparing different sets of data, as in differential film
response to different isotopes, or to the same isotope in different
media (e.g., Alexander et al. 1981; Geary et al., in press).

Expression of Amount of Ligand Bound per Amount of Tissue

The third level of quantification can be achieved if the standards
are calibrated to enable determination of the actual amount of radio-
activity (hence the amount of ligand) present in the tissue. When
the radioactivity per weight of tissue is calculated, the amount of
ligand bound can be easily determined by a formula (Unnerstall et al.
1982) if the specific activity of the labeled compound is known.

Calibration can be achieved in several ways. By sure methods, brain
homogenates with known amounts of radioactivity are both assayed by
liquid scintillation counting and by application to slides for auto-
radiography (Unnerstall et al. 1982; Rainbow et al. 1984). By another
method (Reivich et al. 1969), a brain containing a homogeneously
distributed labeled compound, such as methylglucose or antipyrene,
is sectioned at the midline--half the brain is homogenized for scin-
tillation counting of radioactivity, and the other half is sectioned
and exposed to film with a set of standards covering a wide range of
radioactivity levels. After the counting efficiency is determined
for the machine and fluor used, the OD of the brain sections of known
radioactivity calibrates the standards against brain.

If the isotope is ^{14}C or ^{125}I, all brain regions will show approxi-
mately equivalent ODs if the labeled compound is homogeneously dis-
tributed (Orzi et al. 1983; Herkenham and Sokoloff 1984). If the
isotope is ^{3}H, however, the differential quenching across brain re-
gions will produce a heterogeneous image (figure 1). "Efficiency"
factors can be assigned for each structure based on a comparison with
a weighted average (Unnerstall et al. 1984). Alternatively, the
tissue can be defatted to eliminate the differential quenching (Herk-
enham and Sokoloff 1984; Geary et al., in press), but then two oppos-
ing factors must be assessed: the increased autoradiographic effi-
ciency and the loss of label that occurs by defatting (Kuhar and
Unnerstall 1982). Unfortunately, it is quite difficult to measure
each factor independently. For example, a defatted tissue standard
cannot be prepared without some loss of label in the preparation.
Furthermore, it is difficult to measure radioactivity that is fixed
in tissue. We can estimate it by comparing scintillation counts of
radioactivity in tissue that is unfixed, fixed, or fixed and defatted.
To best extract counts from radiolabeled ligand that is fixed in the
tissue, 1 ml tissue solubilizer (e.g., Protosol, New England Nuclear)
is added to sections in scintillation vials, and these are shaken
overnight. Then 250 µl methanol are added, to reduce chemilumines-
cence, followed by 10 ml of a nondetergent fluor (e.g., Econofluor,
New England Nuclear).

**Determination of Number of Receptors per Weight or Volume of
Tissue**

The "ultimate" in quantification is the determination of the actual
number of receptors per volume of tissue, per cell, or even per synapse.
A number of issues pertain to this level of quantification, including
interpretation of binding data and the rather elusive definition of
of what is meant by a "receptor."

The previous section discusses the third level of quantification and establishes the requirements for determining the amount of ligand bound per amount of tissue. In order to relate the amount of ligand bound to number of receptors, one must determine the percent occupation of the receptors under the in vitro binding conditions used. Using slide-mounted sections which bound ligand is quantified by scintillation counting, the duration of incubation required to reach an equilibruim state should be determined. Next, an analysis of binding saturation, by incubating in different concentrations of ligand, will reveal both the affinity of the ligand for the binding site (Kd) and the number of available binding sites (Bmax).

The qualification that must be kept in mind is that Kd and Bmax are numbers that can change with binding conditions. Often binding is increased by pre-incubation, changes in incubation temperature, or the addition of ions, allosteric effectors and protease inhibitors. Sane binding increases take the form of Kd reductions and others, Bmax elevations. A change in Bmax is not always easy to interpret. Unmasking binding sites by removing endegenous ligand is commonly suggested, but "cryptic" binding sites may be made available by some of the treatments (e.g., Rouot et al. 1980; Chang et al. 1983).

Since most ligands bind to more than one site, altered binding conditions may raise the affinity for a low-affinity site, resulting in complicated Scatchard plots requiring sophisticated analysis. When the binding site is coupled to other molecules, such as ion channels, second messengers or enzymes, binding data can be altered by incubation conditions. In vitro binding conditions used to bring about these effects become far removed from the in vivo environment in which the receptor normally exists. There large chasm between what is known about the action of the receptor in vitro and what it might be doing in vivo.

Some sobering observations about receptor localization and function are already emerging. These take us the full circle, from "absolute" quantification of receptor number, back to the mere localization of receptors to some brain areas and not to others. A striking finding in nearly all neurotransmitter/receptor systems examined to date is that the locations of high-affinity binding sites, which are the easiest to identify autoradiographically, do not necessarily correspond to the areas of the brain that contain the terminations of pathways that release the transmitter for that receptor: there may be no correlation at all between receptor distribution and transmitter distribution for any given putative transmitter-receptor system (see Herkenham and McLean, in press,for review); and, in some cases, there may even be a negative correlation (e.g., Goedert et al. 1984). This puzzling situation has emerged for receptors to transmitters including peptides, catecholamines, indolamines, and the amino acids.

Specific examples of the mismatches are abundant. In the opiate system, it is classic knowledge that opioid peptides have their highest levels in the globus pallidus, a structure which contains only modest numbers of all of the opiate receptor subtypes. Likewise, in the substance P system, the substantia nigra pars reticulata contains

the highest levels of substance P terminal immunoreactivity in the brain (see Mclean et al. 1985); but, in the pars reticulata, substance P receptors are not detectable, and binding for a putative substance P receptor subtype, marked by the analog eledoisin, is barely detectable (figure 4). Conversely, areas that contain little or no demonstrable substance P immunoreactivity, like the hippocampus and cerebral cortex, have very dense concentrations of receptors. One doesn't need quantitative autoradiography to appreciate such profound mismatches!

In our enthusiasm to be quantitative we may miss some of the truly remarkable uses of "quantitative" autoradiography. For example, by examining just the patterns of substance P and eledoisin binding, one might be at a loss to explain why these were ever considered to be related peptides. Pattern recognition is one of the most powerful uses of autoradiography. In a recent tudy by Rainbow et al. (1985), the similar patterns of [^{3}H]MPTP and [3H]pargyline binding supported the suggestion that MPTP produces parkinsonian effects after conversion to a toxic ion by monoamine oxidase (Markey et al. 1984).

Another, perhaps more striking, mismatch can be found in the deep cerebellar nuclei, which are known to be the main sites for relaying cerebellar outputs. For many years physiologists have known that a main feature of cerebellar outflow is an inhibitory connection between Purkinje cell axons and cells of the deep cerebellar nuclei. Subsequently, it was shown that this postsynaptic inhibition is produced by the actions of the neurotransmitter, GABA (Obata et al. 1967), and that Purkinje cells (Pibak et al. 1978) and their terminals (McLaughlin et al. 1974) are immunoreactive for the GABA-synthesizing enzyme, GAL Finally, deep cerebellar nuclei are known to contain moderately high levels of GABA (Fahn and Coté 1968). Given these facts, one would expect to see high levels of GABA receptors in the deep cerebellar nuclei. However, autoradiography of [3H]muscimol binding, produced by in vitro conditions that selectively label "synaptic GABA receptors" (Penney et al. 1981), does not reveal any detectable binding in the deep cerebellar nuclei of the rhesus monkey (figure 5a).

Recently a class of low-affinity GABA receptors has received much interest because it appears to be the class that interacts with benzodiazepines and barbiturates and to mediate physiological responses to GABA (Olsen et al. 1984b; Tallman and Gallager 1985). This class can be marked for autoradiography by the GABA antagonist [^{3}H]bicuculline methochloride (Olsen et al. 1984a). Its autoradiographic distribution matches that of benzodiazepine receptors (Wamsley et al. 1984) and differs somewhat from that of [^{3}H]muscimol. Autoradiography of [^{3}H]flunitrazepam binding, under conditions which would label the benzodiazepine/low-affinity GABA receptor complex (O'Neill et al. 1984), reveals detectable binding in the deep cerebellar nuclei (figure 5b), though it is still quite low in relation to binding in the cerebellar and cerebral cortices. Interestingly, the presence of [^{3}H]flunitrazepam binding in a region where there is no detectable
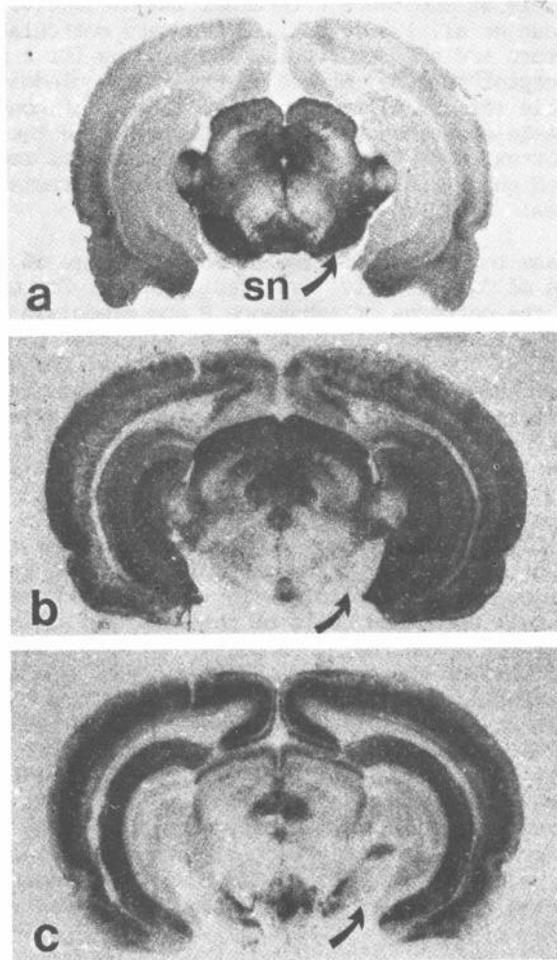
FIGURE 4

Substance P

Autoradiographic film images at the level of rat substantia nigra.
a) Substance P immunoreactivity marked by iodinated antibodies
(McLean et al. 1985) is extremely dense in the nigra and represents
terminations of substance P containing striatonigral projections.
Autoradiograph courtesy of S. McLean. b) Substance P receptors
marked by iodinated substance P (Rothman et al. 1984b) are undetec-
table in the nigra. c) Similarly, eledoisin, a substance P analog
which may bind to a subtype of the substance P receptor (the SP-E
site), has only barely detectable binding in the nigra (Rothman et
al. 1984a). Mismatches of substance P terminals and receptors
are apparent in the cortex as well.
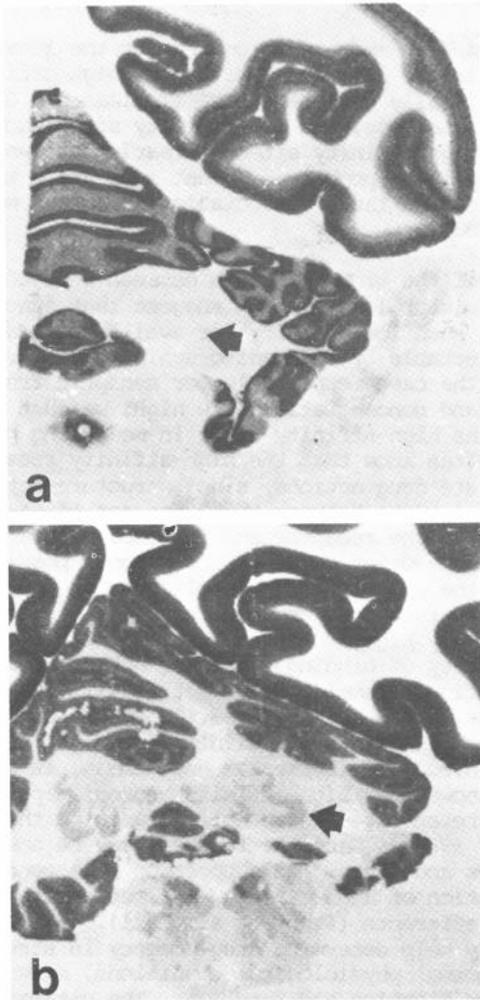
FIGURE 5

GABA

Autoradiographs at level of monkey deep cerebellar nuclei (arrows point to Location of dentate nucleus) a: The binding of 50 nM [^{3}H]muscimol, under conditions that Label the high-affinity GABA receptor (Penney et al. 1981), is undetectable in the deep cerebellar nuclei, which are known to receive dense and functional GABA-ergic inputs. Autoradiograph courtesy of R.P. Hammer, Jr. b: The binding of 1 nM [^{3}H]flunitrazepam, under conditions that label the benzodiazepine/low-affinity GABA receptor complex (see text), is low but detectable in the deep cerebellar nuclei. Autoradiograph courtesy of J.B. O'Neill, D.P. Friedman, and J.M. Crawley.

25

[³H]muscimol binding appears to contradict the idea that the low-affinity site is the same receptor as the high-affinity site, but in a conformationally different form (Tallman and Gallager 1985). For this to be the case, the low-affinity sites should comprise a subset of the high-affinity sites. Clearly more work needs to be done on this exciting problem. Recent indication that the sites are different comes fran their dissociation in mutant mouse cerebellum (Fry et al. 1985).

Such examples of the gross mismatch between receptor localization and neurotransmitter localization suggest that functional receptors may exist with such low affinity for available ligands that they would be undetectable by autoradiographic anaylsis. While this may indeed be the case, especially for synaptic transmission where endogenous ligand concentrations are high, we must still consider the roles of the high-affinity sites in mediating pharmacological effects. We often known that the high-affinity receptors are the ones that mediate drug actions, since structure-activity studies show good correlations between the potencies of analogs to inhibit ligand binding to the receptor and to produce physiological or behavioral effects. Other observations suggest possible nonsynaptic functions for the apparently mislocalized high-affinity sites. A recent ultrastructure study showed that in rat striatal patches, which contain very high densities of high-affinity μ opiate receptors, the large majority of binding sites are not located at synapses as classically defined (Hamel and Beaudet 1984). Thus, nonsynaptic nerve transmission can be suggested. The possibility that transmitters can diffuse through extracellular spaces to reach distant receptors has been discussed (Herkenham and McLean, in press). Another recent study shows that high-affinity benzodiazepine receptors have a very rapid turnover rate--their half-life is on the order of hours (Borden et al. 1984). Receptor turnover may be amenable to regulation by transmitters and drugs. In support of this concept, studies have shown upregulation of high-affinity [³H]muscimol binding after removal of GABA-ergic afferents (Penny et al. 1981). While receptor affinity and specificity help determine drug potency in structure-activity tests, under normal physiological conditions, many factors intervene between binding kinetics and function. The ultimate value of guantitative autoradiography may not be to discover how many receptors there are in a given area, but rather to help elucidate the mechanisms by which drugs drugs and transmitters use and regulate the receptors, and thus reveal dynamic brain processes.

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Brain-Stimulation Reward: A Model for the Neuronal Bases for Drug-Induced Euphoria

Conan Kornetsky

Not in short supply in the field of drug abuse are theories. A
National Institute on Drug Abuse (NIDA) monograph (Lettieri et
al. 1980) entitled "Theories on Drug Abuse" presented 43 separate
essays, each describing a theory attempting to explain the
nonmedical use of drugs. For the most part, with some
exceptions, the theories explicitly stated or implied that use is
engendered only in those individuals who are in sane way deviant
This deviation may be in the realm of personality, biology, or
social systems. Thus, the drug is used to restore one to a
normal state. I believe that this point of view was eloquently
stated by Beckett (1974), ". . . that heroin addiction in some is
symptormatic of an underlying chronic depression in a wounded
personality . . ." Khantzian (1980) argued ". . . that opiates
counteracted regressed, disorganized, and dysphoric ego states
associated with overwhelming feelings of rage, anger and related
depression." Dole and Nyswander (1967, 1980), in introducing the
use of methadone as a treatment modality, argued for the
existence of a metabolic disturbance in the narcotic addict and
that the drug use is restorative.

An argument could be made that these theories are probably all
correct to sane degree. Certainly depressed individuals might
find opiates or cocaine helpful in relieving their depression.
Opiates will decrease aggressive behavior so that we can assume
it decreases feelings of aggression. Methadone certainly will
restore an individual who, as Dole and Nyswander (1980) have
argued, developed a long lasting metabolic deficiency from use of
narcotic drugs. Certainly a depressed existence can become more
tolerable by the use of drugs that elevate one's mood. However,
drug seeking behavior would not exist and none of these putative
restorative states would happen if in some way the abused sub-
stances did not cause some feeling of well-being or euphoria.

Despite these multiple causative factors, this cheer will pre-
sent evidence from my laboratory, as well as others, that there
may exist a common underlying neuronal action of many abuse
substances. At the level of the CNS, it is manifest as an
activation of those areas of the brain for which electrical

30

stimulation is rewarding, and for the user it is translated into some pleasurable feelings that have been described as the "high' In an animal the effect is sufficiently reinforcing that the animal will work to receive the drug or it will make the animal more sensitive to rewarding brain stimulation.

The. possibility that abuse substances achieve their reinforcing value by activating a reward system in the brain was suggested not too many years after the original discovery of Olds and Milner (1954) that animals will work in order to receive such brain stimulation. The phenomenon has been called intracranial self-stimulation (ICSS) as well as brain-stimulation reward, For the most part, but not exclusively, the former term has been used when the animals are in an operant paradigm in which the dependent variable is rate of response. Early investigators, including Olds, saw in the technique a method for the study of the effects of drugs on brain function. The first report of the effects of drugs on rewarding brain stimulation was a 1956 paper published in Science by Olds et al. describing the effects of chlorpromazine and reserpine. In 1957, Killam and coworkers reported at a meeting of the American Society for pharmacology that 10 mg/kg of pentobarbital slightly increased the rate of responding in rats with electrodes in some areas, but not all, of the hypothalamus. Also, they reported that amphetamine caused an increase in response rate as well as a possible lowering of the reward threshold. The inference was that rate of response increased because the brain stimulation became more rewarding.

Olds and Travis (1960) described a series of experinmts in which they studied the effects of morphine as well as a number of other drugs. The most common effect they observed with morphine was a decrease in response rate. Of interest is that the first published paper I could find on the effects of opiates after the Olds and Travis report was published in 1972 (Adams et al.). A more detailed description by this group was given by Lorens and Mitchell (1973). They, as well as other investigators using rate of response as the dependent variable, found that after low doses of morphine rate of response was first decreased followed by facilitation 2 to 3 hours after drug administration. They also reported that after a relatively short period of chronic treatment there was no decrease in rate of response, and significant facilitation in self-stimulation was observed during the second hour after morphine administration.

A further indication that moraine caused an activation of the brain pathways involved in intracranial self-stimulation was given in the early 1970s (Nelsen 1970; Nelsen and Kornetsky 1972). Rats prepared with two bipolar electrodes, one in the medial forebrain bundle-lateral hypothalanus area and one in the brainstem, showed a dissociation in the EEG recorded simul- taneously from these two brain sites after morphine administra- tion. In the former, a positive reinforcing site, there was an activation as reflected in a shift from high to low amplitude waves. At the same time, the EEG recorded from the brainstem, an

aversive site, showed the opposite -- a shift from low to high
amplitude waves. An example of this effect is shown in figure 1.

Earlier, Stein and Ray (1960) reported that d-amphetamine lowered
the threshold for ICSS using a two-lever reset method. In this
procedure the animal has two response levers available. A
response on one lever results in rewarding brain stimulation, but
each response on this lever results in a fixed decrease of
intensity of stimulation in a step-wise manner. A response on
the second lever resets the intensity of stimulation available
from the first lever back to its original intensity. Threshold
is defined as the average intensity at which the animal will
press the reset lever. Stein and Ray clearly replicated the
earlier study by Killam et al. (1957).

Throughout the 1960s and into the 1970s, ICSS was not used to study to any great degree the effects of abuse substances and, as mentioned, opiates were not studied at all during this period.

This failure to evaluate the action of abuse s&stances on ICSS was probably not surprising, considering the early equivocal results with morphine and the emergence in the 1960s of the drug self-administration procedure (Weeks 1962) and the use of drugs as discriminative stimuli (Overton 1964). The drug self-&minis-tration procedure clearly had face validity for the studv of abuse substances, and the drugs as discriminative stimuli pro-cedures, in many ways, is an animal analogue of the Drug Addiction Research Center's (Lexington, KY) method of determining the abuse potential of new compounds in man. Although both of these methods are useful, they do not directly tell us anything about the neuronal bases for the reinforcing effect of a drug-only whether or not it is reinforcing.

As previously stated, there was a resurgence in the 1970s of the use of brain-stimulation reward as a model for the study of the euphoria caused by abuse substances. In 1974, Marcus and Kornetsky demonstrated the functional significance of our earlier EEG; findings (Nelsen and Kornetsky 1972); see figure 2. In this experiment, it was found that morphine lowered the threshold for
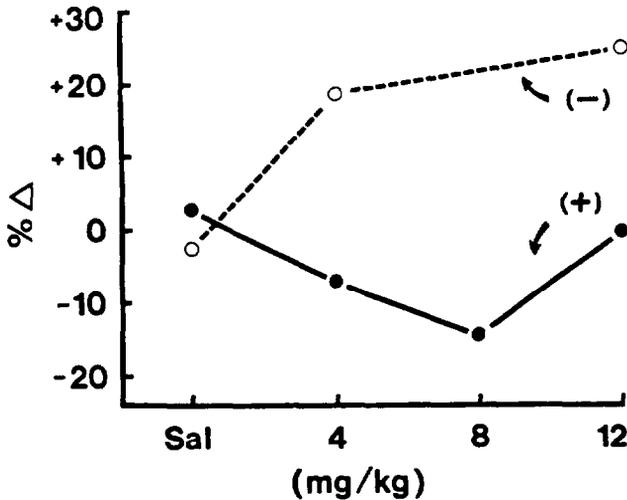

FIGURE 2

Effects of morphine on rewarding (+) and aversive (-) stimula-tion. Ordinate is percent change (%Δ) in threshold from pre- to post-injection (POST-PRE/PRE x 100). Each line represents the mean threshold chanqe for three subjects each on escape and reward thresholds. Adapted from Marcus and Kornetsky (1974). (From Kornetsky and Wheeling 1982b)

rewarding stimulation and raised the threshold to aversive stimu-
lation. Of importance was that it was the first classical
psychophysical study of ICSS that measured the threshold in a
rate (of response) free paradigm Threshold was defined as that
intensity of stimulation which half the time the animal would
find reinforcing and half the time it would be nonreinforcing.
Also, it was the first clear demonstration that morphine lowers
the threshold independent of any stimulating motor effects.
Although a "double staircase" (Cornsweet tracking method of
intensities was used in this experiment, subsequently we have
used the psychophysical method of limits in our studies.

The behavioral paradigm that we use is schematically presented in
figure 3. The procedure allows us, in a manner of speaking, to
ask the animal after the first stimulus (S1), "if you wish you
may have another stimulus exactly the same as the first stimulus
by turning the cylindrical manipulandum." By varying the
intensity of the stimuli according to a psychophysical schedule
of stimuli presentations we can determine the intensity at which
half the time the animal says "yes" to our question and half the
time it says "no."

Figure 4 illustrates a pre- and post-drug power-function after
the administration of 4 mg/kg of morphine. Plotted is the
percent of times the animal decided to respond at each intensity
of stimulation. As can be seen, a shift to the left indicates
increased sensitivity with the threshold defined as the intensity
at which the animal responded 50 percent of the time.

Thus far in our studies we have yet to find a false positive,
that is, a drug that lowers the threshold for rewarding brain
Stimulation that is neither an abuse substance nor a drug that
has been demonstrated to cause euphoria in humans. However, we
have found false negatives, that is drugs that are abused but do
not lower the threshold for reward. As might be expected, the
opiates that are abused lower the threshold, as do amphetamine
(Esposito et al. 19801, cocaine Esposito et al. 1978), and
phencyclidine (Kornetsky et al. 1980). Abuse substances we have
studied that have no threshold lowering effect are alcohol
(Unterwald et al. 1984), pentobarbital (unpublished), and
lysergic acid diethylamide (unpublished). If a complete dose
response curve is generated for a drug that lowers the threshold
for reward, the curve is U-shaped. This is illustrated in figure
5, which shows the dose-effect relationship after the administra-
tion of d-arqhetamine. Since the curve is U-shaped, the maximum
effective dose for an individual animal or animals can be
determined.

After our initial success with demonstrating that a number of
drugs lower the threshold when abused, we expanded our studies to
mixed agonist-antagonist opioids. We tested threeofthese:
cyclazocine, nalorphine and pentazocine (Kornetsky et al. 1979).
Of these three drugs, only pentazocine consistently lowered the

threshold. However, the extent of this lowering (maximum efficacy) did not reach the level observed after morphine, cocaine, or d-amphetamine. The relative threshold lowering effect of these mixed agonist-antagonist drugs as well as morphine, cocaine, and d-amphetamine is shown in figure 6.
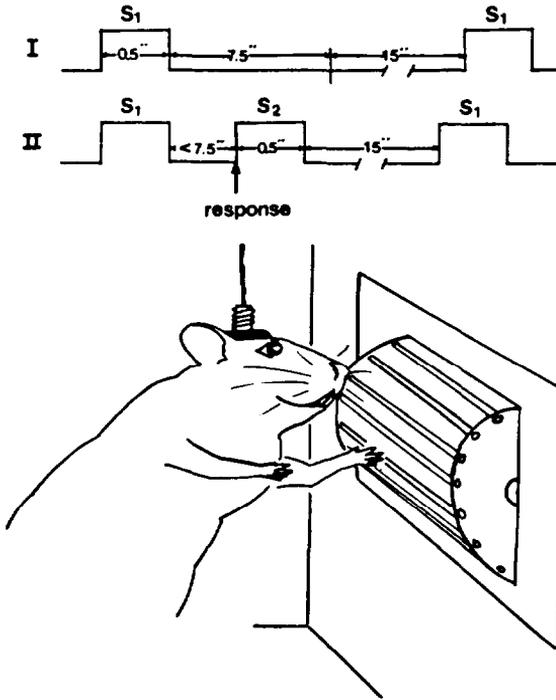
FIGURE 3

A schematic representation of the method for determining brain-stimulation reward thresholds. (I) A sequence of a single trial when an animal doss not respond. (II) The sequence when the animal makes a response. A trial begins with the delivery of a non-contingent stimulus train (S1); if the animal fails to respond by turning the wheel within 7.5 seconds from the onset of the S1, the trial is terminated and 15 seconds later the next trial is begun. If the wheel is turned as indicated in example II, the rat receives a contingent stimulus (S2). Intensity of stimulation is varied according to a modification of the method of limits. A response after the 7.5 second available response time postpones the presentation of the next stimulus for 15 seconds. (Fran Kornetsky and Bain 1982)
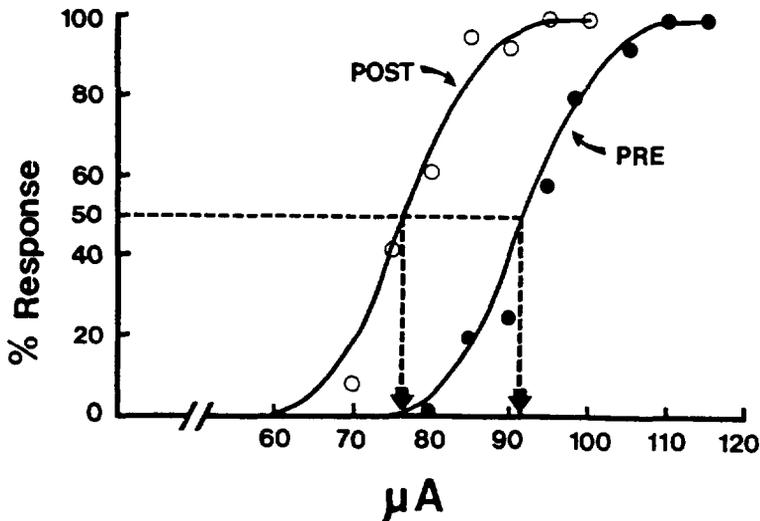
FIGURE 4

The effects of 4 mg/kg of morphine (subcutaneous) on the threshold for brain-stimulation reward. The percent of trials in which the subject responded at each current intensity are presented. PRE and POST data were obtained immediately prior and subsequent to drug injection. Thresholds are indicated as the median rewarding intensities at which the subject responded. (From Kornetsky and Wheeling 1982a)

Also shown in figure 6 is the effect of phencyclidine (PCP). Of the five animals studied, only in one did PCP not lower the threshold. However, as can be seen, the extent of this threshold lowering effect is slight and approximates the same level established by pentazocine.

In order to test the model further, we decided to examine a common street combination of drugs, tripelennamine and pentazocine (called "T's and Blues" on the streets). As described above, we had already demonstrated that pentazocine had significant but relatively low efficacy in lowering the threshold. Before studying the combination, we Qtermined the effect of tripelennamine alone on the threshold for rewarding intracranial stimulation. To our surprise we found that tripelennamine significantly lowered the reward threshold (Unterwald et al. 1984b). However, this finding could have been predicted from the experiments of Jasinski et al. (1983) who found that human subjects given tripelennamine alone report an increase in euphoria as measured by the Addiction Research Center Inventory. The effect of tripelennmine alone is shown in the
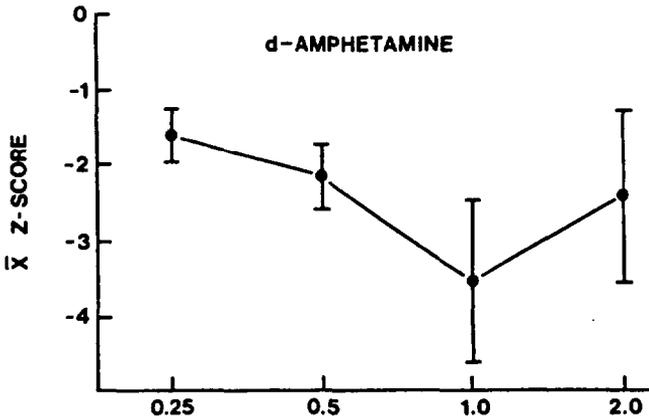
FIGURE 5

Mean Z-score changes in threshold after various doses of d-amphetamine. Z-scores are based on the post-pre changes in threshold after drug as a function of the $\bar{X}$ and standard deviation of post- pre-changes in threshold after saline for each animal. Z-scores are conputed as follows:

$$\frac{\text{Saline } \bar{X} \text{ (post - pre) - treatment threshold (post - pre)}}{\text{Standard deviation of saline } \bar{X}}$$

Thus changes of 2 indicate the 95% confidence limits. Error bars indicate the standard error of the Z-score.
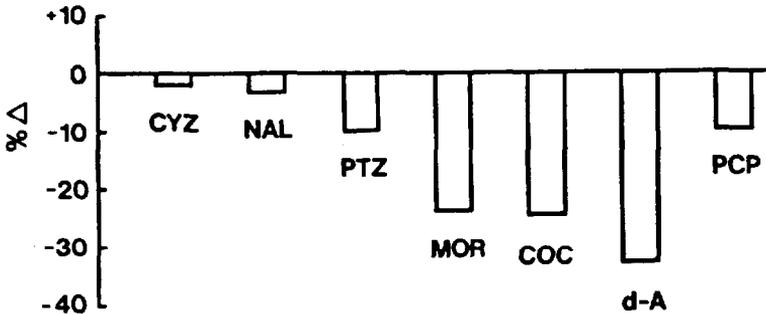
FIGURE 6

Median effects of various drugs on brain-stimulation reward (POST-PRE/PRE x 100) based on the optimal lowering of threshold, independent of dose. Negative scores indicate the extent of effect below the maximal decreases obtained with vehicle. CYC: cyclazocine, N=4: NAL: nalorphine, N=4; PTZ: pentazocine, N=4; MOR: morphine, N=10; CCC: cocaine, N=7; d-A: d-amphetamine, N=4; PCP: phencyclidine, N=8. (From Kornetsky and Wheeling 1982a)

the upper graph, and the lower graph shows the effects of
pentaxocine alone and these same doses in combination with an
ineffective dose of tripelennamine (0.625 mg/kg).

As can be seen, not only is the dose effect curve moved to the
left, but the maximum effect is clearly greater with the combina-
tion than with any dose of pentazocine alone. Also, the effect
of the combination is greater than the effect of any dose of
tripelennamine alone.

We tested one other mixed agonist-antagonist alone and with
tripelennamine. This drug, nalbuphine, is currently only
available in injectable form for clinical use as an analgesic.
Jasinski and Mansky (1971) reported that it has significantly
less abuse potential than morphine. An example of the type of
effect of this combination in one animal is given in figure 8.
As can be seen, not only does nalbuphine lower the threshold but
the interaction with tripelennamine is similar to that seen with
the tripelennamine and pentazocine combination

The results of this combination suggest that if nalbuphine
becomes generally available, it may be used in combination with
tripelennamine as pentazocine currently is. However, it has been
reported that nalbuphine has greater efficacy as an opiate
antagonist than does pentazocine Schmidt 1983). Thus, in the
morphine or heroin dependent individual, the use of nalbuphine
may precipitate abstinence which would preclude its use.
However, in the nordependent individual the combination clearly
has abuse potential.

The question that these experiments raise is whether or not there
is a common neuronal substrate for these threshold lowering
effects. Most investigators believe that the underlying neuro-
chemical substrate is catecholaminergic with heavy emphasis on
the dopamine system (e.g., Fibiger 1978; Wise and Bozarth 1982;
Gallistel et al. 1981; Olds and Forbes 1981). However, recently
Prado-Alcala and Wise (1984) and Prado-Alcala et al. (1984) have
found that there is not a close correspondence between the
boundaries of the brain reward system and those of the dopamine
terminal fields. These findings suggest that a direct activation
of a dopaminergic system does not account for the rewarding
effects of stimulation to a variety of brain sites.

The facilitation of the threshold lowering effect of the mixed
agonist-antagonists by the antihistamine tripelennamine suggests
that a central histamine system may also play a role in the rein-
forcement effects of opiate drugs. Belluzzi and Stein (1977)
have suggested a role for an endorphin system in brain-stimula-
tion reward. This is based, for the most part, on their findings
that naloxone will block or attenuate brain-Stimulation reward.
Although some investigators also have found that naloxone will
attenuate ICSS behavior (Stapleton et al. 1979; Schaeffer and
Michael 1981), other investigators using similar procedures with
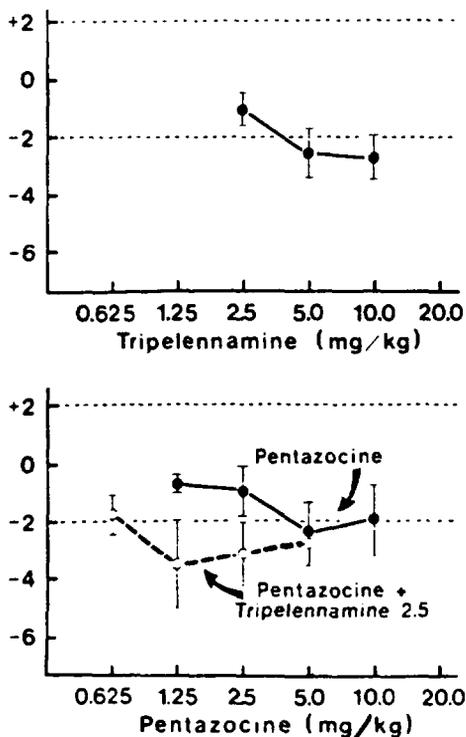
FIGURE 7

Mean Z-score effects of various doses of tripeleunamine (top
panel) and pentazocine (bottompanel). The bottom panel also
shows the effects of 2.5 mg/kg of tripelennamine with various
doses of pentazocine. Error bars indicate the standard error of
the $\bar{X}$ Z-score. A Z-score of ± 2 indicates the 95% confidence
limits. (Fran Unterwald and Kornetsky 1984)

electrodes in similar brain sites have failed to find an effect
of naloxone Wauquier et al. 1974; Holtzman 1976; van der Kooy et
al. 1977; Lorens and Sainati 1978).

Stapleton et al. (1979) reported that relatively large doses (10
mg/kg) of naloxone did not abolish self-stimulation but caused
relatively small reductions in lever-press rate. They concluded
that endorphinergic processes my modify reward but did not seen
to be critical for reward We have found that doses of naloxone
up to 16.0 mg/kg, both acutely (Esposito et al. 1980) and chroni-
ically (Berry et al. 1981) administered, had no effect on the
reward threshold in animals with electrodes in the medial fore-
brain bundle (MFB) or ventral tegmntal area (VTA) except to in-
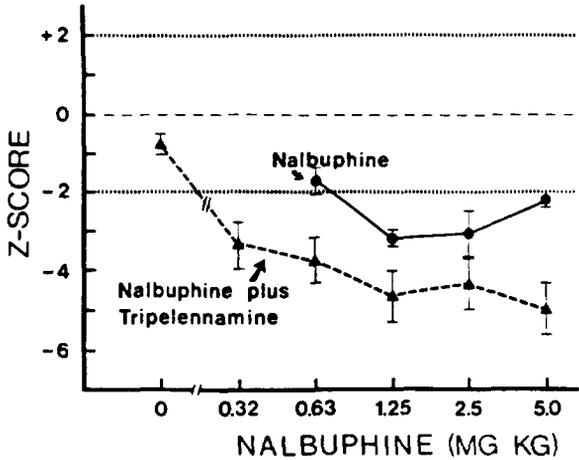
FIGURE 8

Mean effect of various doses of nalbuphine alone and in
combination with an ineffective dose of tripelennamine.

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crease within subject variability. We have, however, found that
naloxone at doses of 2.0 to 4.0 mg/kg will reverse the effects
of 0.5 or 1.0 mg/kg of d-amphetmine. Higher doses of naloxone
often did not block as well as the 2.0 to 4.0 q/kg. The effects
of naloxone on the threshold lowering effect of d-amphetamine, as
well as cocaine and morphine, are shown in figure 9. Note that

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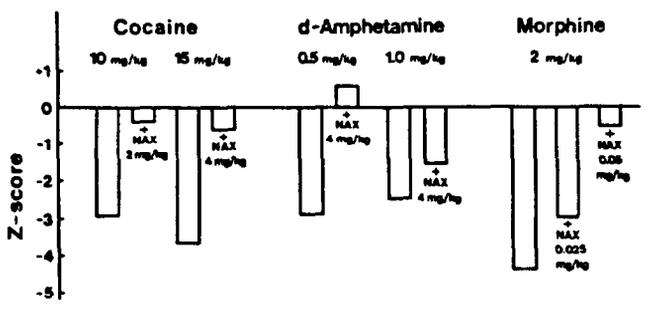
FIGURE 9

Effects of various combinations of naloxone with cocaine, d-
amphetamine, or moprphine on brain-stimulation reward threshold.
Z-scores are based on the mean and standard deviation of thres-
hold changes (POST-PRE) after respective vehicle injections. Z-
scores beyond ± are considered significantly different Data are
based on the man Z-score for 6 subjects with cocaine, 4 subjects
with d-amphetamine, and a single morphine subject. The naloxone
block or attenuation of the respective drug effect was observed
in all animals. (Fran Kornetsky and Wheeling 1982a)

there is a tremendous difference in potency between naloxone's effect compared to its ability to block cocaine or d-amphetmine.

These results support the hypothesis that there may be endorphinergic receptors on the presynaptic dopamine neuron which serve as modulators of the dopamine system and thus may be part of a common neuronal substrate involved in the euphoria caused by the narcotic analgesics as well as cocaine and d-amphetamine. Contrary evidence, however, is given by Pettit et al. (1984). They found that destruction of dopamine terminals in the nucleus accumbens of the rat would selectively attenuate cocaine but not heroin self-administration. This discrepancy in interpretation of our findings using brain-stimulation reward and the Pettit et al. findings using self-administration suggests that the two models may not be measuring the same phenamenon. Only future research will solve this apparent contradiction in findings.

A major question is whether or not drugs cause differential effects as a function of where the stimulating electrodes are implanted. Ever since the first drug study of the effects of drugs on ICSS by Olds et al. (1956), there have been attempts to use this technique to determine site of action of the drugs. These attempts have been criticized on procedural grounds primarily because there has been a confounding between rate of response and threshold. Most of the site specificity studies have used rate as the dependent variable or a rate dependent threshold procedure. Many investigators have found differential effects of drugs as a function of site of stimulation. This has been especially true for studies with d-amphetmine (e.g., Carey et al. 1975; Franklin and Robertson 1982; Goodall and Carey 1981) and opioids (e.g., Liebman and Segal 1977; Lorens 1976; Nazzaro et al. 1981). The major difficulty with these studies is that the sites themelves seem to engender different response rates. Even if response rates were equated, as Esposito and Kornetsky (1978) and Liebman (1983) point out, response rates may be at a ceiling at one placement and not at another placement. Also, if baseline response rates are not equated there is still the problem of "rate-dependency." Further, Schenk et al. (1981) did a within subject comparison of the effects of morphine on lateral hypothalamic and central gray self-stimulation In addition, they were also able to compare between subject differences. They found that the time course and magnitude of drug-caused changes in effects in the two placements was a function of individual animal differences, not electrode placement differences.

As mentioned previously, the baseline rates of responding will differ as a function of site of electrode placement It is not even clear whether or not these rate differences directly reflect the reward value of the stimulation Over 20 years ago Hobos and Valenstein (1962) found, in a paradigm involving two electrodes associated with two levers respectively, that animls did not necessarily choose the lever that resulted in stimulation to a site that generated the highest rates. Although the number of subjects was small, Esposito et al. (1979) compared the effects

of morphine on the threshold for rewarding stimulation in animals
with electrodes in the VTA, the ventrolateral portion of the
central gray, or the anterior tip of the locus coeruleus. They
concluded that, at least from this data, it would be premature to
specify a precise neuroanatomical and/or neurochemical substrate
for the observed effects.

We directly compared rate of response and threshold in the same
animals in order to test the hypothesis that rate was inversely
related to thresholds (Payton et al. 1983). Figure 10 presents
the scatter plot from this experiment. As can be seen, there is
no significant correlation. Complete power functions were done
on each animal and the greatest rate of response was the datum
used for an individual animl. Since electrodes were aimed for a
number of structures, a comparison of threshold as well as rate
of response as a function of site of stimulation could be
compared. This is shown in table 1. Although significant
differences in threshold as a function of site were found, no
such differences were found in rate of response. Also, when
rank, from lowest to highest in both threshold and rate is
compared, there is no relashionship between threshold and rate.
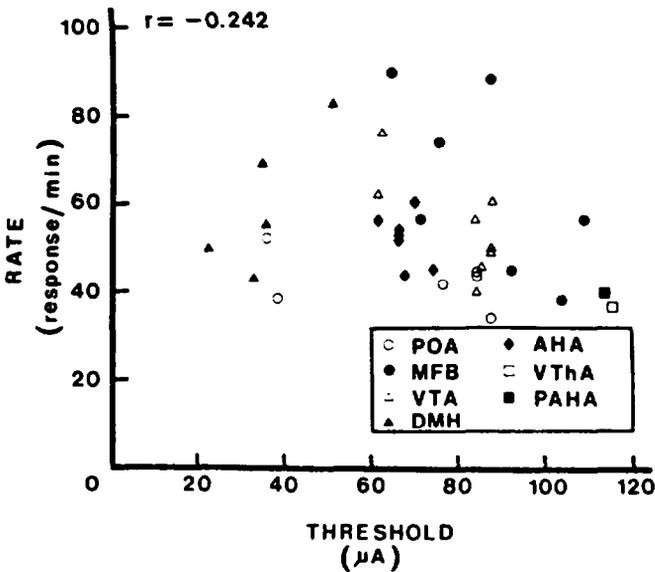
FIGURE 10
Threshold/rate scatter plot, Each point represents a separate
animal. POA: preoptic area: MFB: medical forebrain bundle; VTA:
ventral tegmental area; DMH: dorsal medial hypothalamus; AHA:
anterior hypothalamus; VTHA: ventral thalmic area; PAHA: pre-
optic-anterior hypothalamic area,

Somewhat contrary data are given in the recent reports of Gratton
and Wise (1983) and Prado-Alcala et al. (1984). They did mapping
studies in which they used a dorsal-ventral movable electrode.
They measured rate of response and a threshold which they defined
as the maximum current intensity that sustained 10 or less
responses per minute. They classified the rate data from each
animal as either low, medium, or high. Although they did not
publish the mean threshold obtained at each of the response rate
levels, enough of the data were presented that I was able to
calculate the mean and SD for each level. Going from low rate to
high rate, the mean threshold ± SD was 36.4 ± 10.9, 39.9 ± 10.5,
and 22.8 ± 8.7 µA, respectively. There was a clear difference in
threshold between the high rate sites and either the law or
medium rate sites. Although we found that the highest rate of
response was from the MFB, as did Gratton and Wise, cur finding
that the MFB also yielded the highest absolute threshold was
contrary to Gratton and Wise. This would indicate that their
threshold measure was not independent of rate of response.

TABLE 1

Comparison of Threshold and Response Pates
from Stimulation to 5 Brain Sites

Brain Sites	Threshold* (Rank)** (µA)	Rate of Response* (Rank)** (Response/Min)
Dorsal Medial Hypothalamus	37.7 ± 6.7 (1)	55.6 ± 8.4 (4)
Anterior Hypo- thalamic Area	6 8 . 8±.2 (2)	52.5±2.6 (2)
Preoptic Area	68.9±10.2 (3)	41.9±2.6 (1)
Ventral Tegmen- tal Area	81.3±4.1 (4)	53.7±3.9 (3)
Medial Forebrain Bundle	86.5±6.0 (5)	62.8±7.9 (5)
ANOVA F ratio	9.32 (P<.001)	1.89 (P<.10)

* Mean ± SE.
** From lowest to highest. (If rate reflects threshold then the
   lower the rate the higher the threshold).

The threshold measure we use is in classical psychophysical terms
the absolute threshold. That is, the intensity of stimulation
that is recognized half the time by the subject. The threshold
used in the previously described studies by Wise and his

collaborators, as well as others, is defined as the intensity of stimulation that gives a particular rate of response. These levels are clearly above the intensity at which the animal will respond only half the tine. Thus, the type of threshold may be important.

Interpretation of site of action based on thus locus of the tip of the stimulating electrode can be misleading. We determined the minimum level of stimulation that an animal could detect when the minimum level of stimulation that the animal found rewarding had already been determined (Kornetsky and Esposito 1981). In both types of threshold determination the same electrodes in the same animals were used; thus, the site of stimulation was identical. As might be expected, the detection threshold is well below the reward threshold. We then compared the effects of cocaine on both thresholds. Figure 11 shows the results of this experiment. At doses at which cocaine lowered the brain-stimulation reward threshold it raised the threshold for detection. This dissociation clearly points to the difficulty in attributing a drug effect as being at the tip of the stimulating electrode.
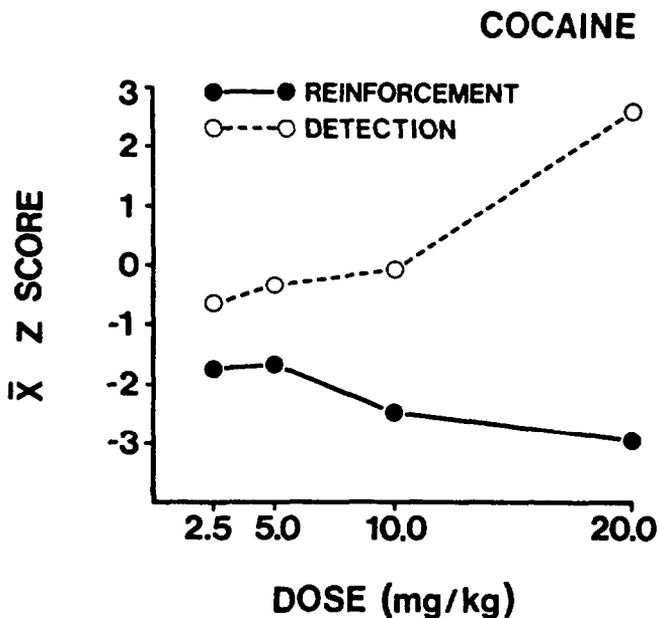
FIGURE 11

Men effect of various doses of cocaine on the threshold for brain-stimulation reward (reinforcement) and on the threshold for brain-stimulation detection, Data are expressed as Z-scores based on the respective mean and standard deviation of the effects of saline N=4). Adapted from Kornetsky and Esposito (1981) (Fron Kornetsky and Rain 1982)

More important, it suggests that the task and the integrated systems involved in the demands of the task are more important in understanding a particular drug effect than is the site of Stimulation per se.

CONCLUSION

Our work has stemmed from the hypothesis that the threshold lowering effect of abuse substances reflects the drugs' ability to cause euphoria in man (Marcus and Kornetsky 1974). This position has been substantiated by many studies by a variety of investigators. Further, it has been established (Esposito and Kornetsky 1977) that tolerance does not develop to the threshold lowering effects of morphine. This suggests that physical dependence is not necessary for the rewarding effects of drugs (Kornetsky and Esposito 1979; Kornetsky et al. 1979). Recently, Bozarth and Wise (1984), in an experiment in which animals were trained to press a lever for microinjections of morphine into the ventral tegmental area, found no signs of physical dependence, but physical depedence was seen after similar infusions into the periventricular gray region

Finally, strong support for the role of the reward system in the action of abuse substances is found in the experiment by Seeger et al. (1984). This group used the quantitative 2-Deoxyglucose method of Sokoloff (1982) to study the effects of amphetamine on ICSS. Animals were prepared with electrodes in the VTA. They compared four groups of animals: high current, low current plus 0.5 mg/kg of d-amphetamine, 0.5 mg/kg d-amphetanine but not allowed to self-stimulate, and a no treatment group In the high current and low current plus amphetamine groups, response rates for rewarding stimulation was approximately the same. Rates of local cerebral glucose utilization (LCGU) was lower at stimula-tion site and pathway in the low current plus d-amphetamine group than the high current group. However, in a number of the projec-tion areas of the VTA, LCGU's were equivalent, i.e., the nucleus accumbens, the medial frontal cortex, the basolateral amygdala and the locus coeruleus. In the olfactory tubercle and the sulcal cortex, LCGU was greater in the low stimulation plus d-amphetamine group than the high Stimulation group.

Although these are sane of the first drug-brain stimulation studies done by this group of investigators, these findings do support the contention that abuse substances activate functional reward systems and a reasonable assumption is that this may be the basis for the euphoria and reinforcing properties of abuse substances.

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The Neural Systems Approach to Examining the Actions of Drugs on the Central Nervous System

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Donald J. Woodward

INTRODUCTION

One of the goals of the study of drug actions is to create a
continuity of understanding between different levels of
experimental analysis. Current approaches to the problem
range from the quantum chemistry of receptor binding to the
social behavior of human groups. The "neural systems"
approach involves study of the activity of neuronal circuits
within the intact, functioning brain, with the aim of
determining the substrates of relevant behaviors, In terms of
a hierarchy of "levels" of functionality of the central
nervous system, this approach might be considered to occupy
the niche between approaches at the cellular level and the
purely behavioral level, As such, investigations of the
effects of iontophoretic or systemic administration of drugs
on single cells on well-understood brain circuits, and in the
context of well-defined behaviors, are commonly carried out
with the hope of forming a bridge between cellular and
behavioral pharmacology.

Why be optimistic about the ability of the neural systems
approach to bridge these disciplines? Rapid advances are now
being made in describing the anatomy and physiology of the
neural circuits and their roles in the control of behavior.
Furthermore, investigations into the roles of various
neurotransmitters in these circuits are progressing rapidly.
Each advance in these traditional disciplines opens new
possibilities for parallel studies on the mechanisms of action
of drugs of abuse. Our conviction is that the most rapid
advances occur when these studies are planned and executed
together.

Drug investigations at the neural systems level seek to shed
light on the "circuit mechanisms" of their action on the

brain, as distinct from "cellular mechanisms." While it is theoretically possible that an understanding of drug effects on cellular mechanisms alone could lead to a prediction of their effects on overall brain circuits, the circuits' complexity appears to preclude such an outcome. Several factors could contribute to this difficulty in predicting the behavior of neural circuits. For example, a circuit may respond strongly to a drug at the biophysical level, but cause no behavioral change because of internal compensation mechanisms that counteract the drug effect. Alternatively, a drug may have little detectable effect at the biophysical level, but produce a marked behavioral change. This could result from a multiplication of subliminal effects at each relay in a multisynaptic system. In view of these possibilities, it appears that a proper search for the mechanism of drug action must be conducted at the level of the emergent properties resulting from the circuit architecture, in addition to interactions at the biophysical level.

To illustrate how such an approach might be implemented we will briefly summarize some work which has recently been carried out in this laboratory. This involved characterization of the effects of ethanol and other anesthetic substances on single unit discharges in the awake, behaving rat.

ETHANOL AND SENSORY TRANSMISSION

These studies were initiated in an effort to provide information about the circuit mechanisms underlying the potent effects of alcohol and other drugs on sensori-motor and cognitive functions. Human performance studies have shown that some of the lowest dose effects of ethanol involve reductions in the ability to quickly move in reaction to a particular sensory stimulus during simultaneous presentation of distracting stimuli (Moskowitz and Sharma 1974; Levine et al. 1975; Barry 1979). Such a deficit may be a major factor in alcohol-related traffic accidents.

What are the circuit mechanisms underlying these drug effects? It is reasonable to suspect that some components of such "sensorimotor filtering" are normally expressed as part of the feature extractive and/or sensory modulating capabilities of the cerebral cortex. We have approached this problem, therefore, by developing a neurophysiological model in the rat of behavior-linked sensory modulation in the sensorimotor cortex.

It has been shown in several mammalian species (Nelson 1984 [monkey]; Coquery 1978a, 1978b [cat]) that cutaneous sensory transmission through the afferent pathways to, and within, the somatosensory cortex is modulated just before onset and during active movement. This modulation may be caused by direct or indirect actions of motor cortical projections to the primary

somatosensory (SI) cortex (Jones et al. 1978 [monkey]; Chapin
et al. 1981 [rat]), or afferent systems to the SI cortex (see
Towe 1973). Such higher-order control of afferent
transmission may play a role in sensori-motor coordination.

We have investigated such sensory modulation phenomena in
experiments involving single unit recording in the forepaw
area of the SI cortex of awake, moving rats (Chapin and
Woodward 1981, 1982b, 1982c). These experiments (described
below) demonstrated that sensory transmission from the skin of
the paw to certain cortical neurons may be momentarily "gated
out" during reaching movements of the forelimb. This appeared
to result in a division of cortical cells into categories
which selectively respond either to "expected" sensory input
(type A cells) or to "unexpected" input (type B cells). An
example of a type B cell is shown in figure 1. This shows
responses of a single neuron to touch stimuli during rest and
running behavior. Figures IA and 1B show poststimulus time
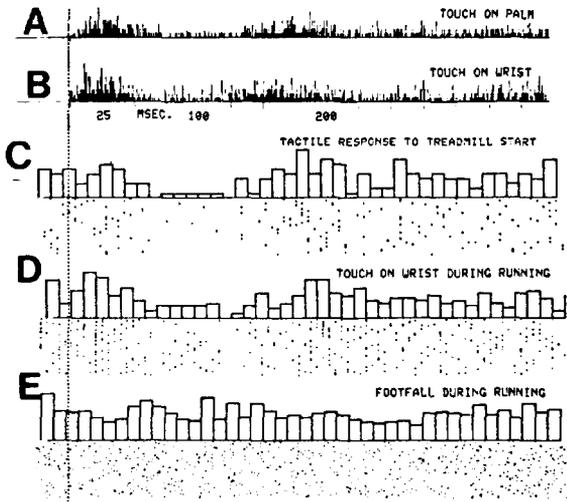
FIGURE 1

Responses of a single SI cortical neuron to passive
and active touch of forepaw. A and B: poststimulus histograms
of unit responses (composed of an early excitatory peak, a
postexcitatory inhibition, and a late peak) to passive touch
of palm and wrist (at dotted line) with a probe (1 msec bins).
 C: perievent histogram showing same unit's sensory response
to sudden start of the treadmill (10 msec bins; same time
scale as in B). D: Unit's response to touching on the wrist
with a hand held probe during treadmill locomotion. E: Unit's
response to contact of the same forepaw on the ground during
treadmill locomotion.

53

histograms of the unit response to probe touch on palm and
wrist respectively (1 msec/bin). The three perievent
histograms below show the response of the same neuron to the
mechanical stimulation of the paw experienced when the
treadmill started (C), to touching the wrist with a hand-held
probe during the swing phase of locomotion (D), and to contact
of that paw on the ground during another locomotor sequence
(E). It can be seen that though the unit did not respond to
footfall (E), it did respond to probe touch of the forepaw
during swing phase (D), and to the mechanical stimulation of
the forepaw caused by the sudden onset of treadmill movement
(C). In type A cells, a roughly opposite pattern was
evident, i.e., they responded to footfall, but not to natural
or electrical stimulation during swing phase.

In order to examine such responses during drug exposure, a
quantitative stimulation paradigm was developed. In these
studies, a small stimulating electrode, chronically implanted
under the skin of the forepaw, was used to quantitatively test
afferent transmission to single units in the SI forepaw area
during different behaviors or during various phases of limb
movement. Using this test, such transmission was found to be
subject to a time-varying, selective inhibition during
treadmill locomotion. Thus, in type B cells, this had the
effect of "gating out" sensory responses to touching the skin
of the paw to the ground at footfall (whose timing is
predictable based on the trajectory of forelimb movement), but
"gating in" responses to stimuli delivered during the swing
phase (when unpredicted obstacles in the running path might be
encountered), Conversely, in type A cells, sensory responses
to skin stimulation during swing phase were gated out, while
responses to footfall were gated in.

Figure 2 illustrates a schematic diagram of the
neurophysiological findings relevant to these two types of
cells, as well as hypothetical mechanisms which may explain
these findings. Schematic perievent histograms (figure 2:A,B-
right, in black) averaged around the footfall (FF) in the step
cycle show the typical responses of these cells to footfall
and to probe stimuli delivered during swing phase. "Perievent
afferent modulation histograms," shown below the perievent
histograms in A and B (labeled "time course of inhibition")
show typical time courses of inhibition (expressed as a
percentage of total possible inhibition) of sensory
transmission over the step cycle for these two types of cells.
Dotted lines above the perievent histograms show how they
would theoretically look in the absence of this inhibitory
gating. Thus, in this model, two different patterns of phasic
afferent inhibition appear able to allow sensory inputs from
the paw to different groups of cells according to whether the
predictable footfall event is imminent or not.
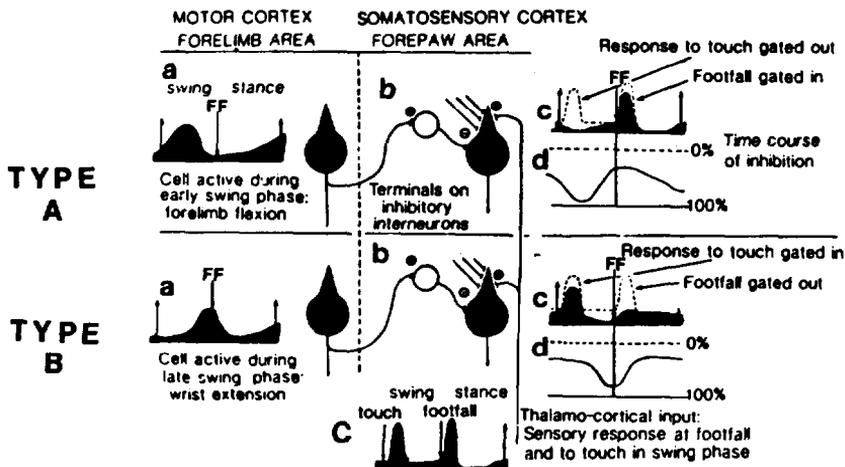
FIGURE 2

Schematic diagrams of hypothetical mechanism of sensory
gating. Perifootfall histogram (C) shows hypothetical
responses of cutaneous afferents (from forepaw) to: 1)
footfall, and 2) probe touch delivered during the swing phase
of the step cycle. In the SI cortex, type A cells are gated
to respond only to the footfall stimulus (see perifootfall
histogram A: c). Type B cells only respond to the touch during
swing phase (B:c). The "perievent gating histograms" (A:d and
B:d) show time course of inhibition of cutaneous afferent
transmission which produces the observed gating effect.
Hypothetical circuit mechanism for the gating (A:b and B:b)
involves axonal projections from neurons in adjacent motor
cortex (dark cells at left), which, through interneurons (open
circles) may inhibit SI cortical neurons (dark cells at
right). Perifootfall histograms in A:a and B:a show observed
activity in different classes of motor cortical neurons.

One hypothesis to explain the observed inhibition is that
this inhibition may result from the activity in the rostrally
adjacent motor cortex of movement correlated units, which have
been shown to project to the somatosensory'cortex. (In figure
2, hypothetical collaterals of motor cortical neurons are
shown inhibiting SI cells through interneurons.) In our
experiments, most such motor cortical cells discharged
strongly either during the middle swing phase or just before
footfall. These time courses corresponded closely with those
of afferent inhibition previously shown in the somatosensory
cortex.

To the extent that this "selective gating" phenomenon
constitutes an example of higher order sensoriwtor processing

in the cortex, it may be of value as a model for investigation
of the effects of alcohol and other drugs which affect
sensorimotor coordinative abilities. Figure 3 shows results
of an experiment which assessed the effects of a low dose of
ethanol (0.3 g/kg body weight, intra-peritoneal) on responses
of a single neuron in the cortex of an awake, freely moving
rat to constant stimulation through an electrode chronically
implanted under the skin of the forepaw. (According to
benchmark experiments, such an injected dose should reach 0.55
to 0.6 g/kg blood concentration between 5 and 10 minutes
postinjection.) The histogram in figure 3A shows the response
of this unit to such stimulation during normal awake, resting
behavior. The initial excitatory responses of such neurons
are often divided into two separate early excitatory peaks
(E1a'and E1b, figure 3A).
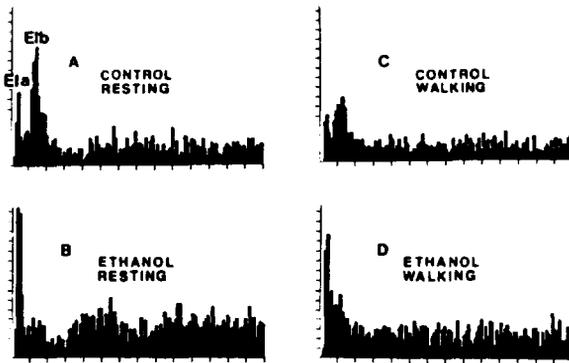
FIGURE 3

Responses of a single SI cortical neuron to paw stimulation
during "resting" (A,B) and "walking" (C,D) behavior before
(A,C) and after (B,D) ethanol administration. Bin width: 1
msec; X-axis ticks: 25 msec; Y-axis ticks: 5 spikes/sec/bin
(normalized for all histograms).

The histogram in figure 3C, which was constructed by using
the same stimulus parameters during treadmill running, shows
that both components of this neuron's response to the same
stimulation during locomotion behavior were markedly reduced.
Five to fifteen minutes after ethanol injection the following
changes occurred: 1) the E1b (late) component of the initial
response was selectively reduced (see figure 3B), and at
higher doses replaced with an inhibition. 2) Though the E1a
(early) response was itself reduced somewhat, it became much
less variable across behaviors. In particular, the level of

this response during movement in ethanol intoxication was actually increased relative to similar locomotor behaviors before the drug injection (compare figures 3C and 3D). Thus, ethanol reduced an inhibitory process which is normally associated with selective gating of sensory transmission in the normal state. Similar, though more profound, effects have been found at higher doses (Chapin and Woodward 1983).

What might be the circuit mechanism of this effect of ethanol on sensory gating? The hypothesis set forth in figure 2 suggests that the gating may result from the activity of movement correlated cells in the rostrally adjacent motor cortex. Figure 4 illustrates the effect of a low dose of ethanol (0.5g/kg) on a neuron isolated in the forelimb area of the motor cortex. As is typical of neurons in this cortical area, this neuron discharged most actively during active movement of the forelimb, specifically the forepaw-projection movement just preceding footfall during locomotion. The strength of this movement correlate was quantitated through the use of a computer synchronized videotape recording of the rat's movements. Each video field-frame (with 17 msec resolution) was labeled with an automatic video frame counter so that subsequent frame-by-frame analysis could be used to correlate movement and unit activity.

The perievent histograms in figure 4 were generated by averaging unit activity around the footfall event (which marked the termination of the movement relevant to this cell). These were constructed for each of 28 l-minute running periods (each containing from 30 to 40 steps) before and after ethanol administration. The intensity of unit firing (in spikes/sec) during the movement-related discharge (marked by "E" in the bottom-left histogram, and the line plot in figure 4), as well as the inhibitory period (I), were measured with the computer and plotted as a percent change from the discharge rate during a control epoch (C) in the histogram. Time of ethanol administration is marked by an arrow at the left of the time axis. Before ethanol administration (see bottom-left histogram), the discharge rate (spikes/set) during the reaching movement averaged about 80% above that measured in the late stance phase (considered as a control period). No inhibition was seen after this excitatory phase. Starting at 3 minutes postethanol, a steady decline in the motor response was measured, and after 11 minutes It was only about 20% above the control level (top-middle histogram). In addition,
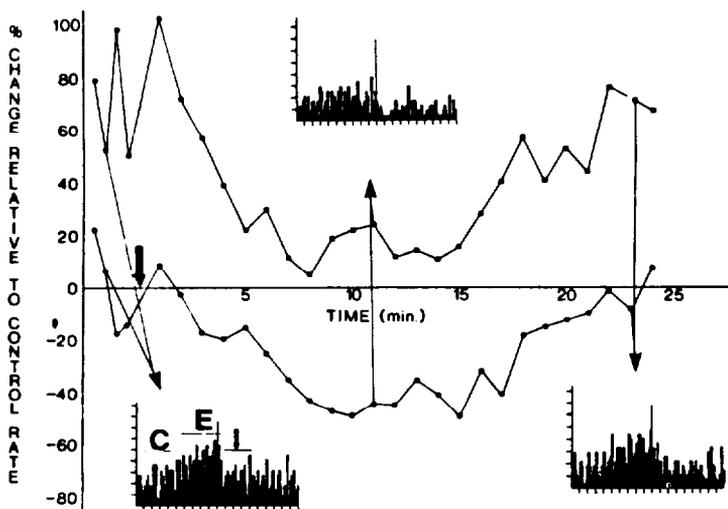
FIGURE 4

Time course of ethanol's effect on forelimb movement
correlated discharge in the rat motor cortex, Perifootfall
histograms: pre-ethanol (bottom-left); 11 mins postethanol
(top-center); 24 mins postethanol (bottom-right). Bin width:
2 msec; X-axis ticks: 50 msec; Y-axis ticks: 10 spikes/set/bin.
See text for further explanation.

an inhibitory period appeared in the postexcitatory phase
(corresponding to early stance phase). This had a time course
similar to that of the reduction in the excitatory response.
Even while these motor cortical responses were drastically
changed only a moderate disturbance of the locomotory gait was
observed. After 20 to 25 minutes (bottom-right histogram),
both excitatory and inhibitory responses recovered to pre-
alcohol levels. From 4 to 20 minutes after ethanol injection,
the normal movement correlated activity of this cell was
markedly reduced or almost abolished. Nearly full recovery
was subsequently achieved. We have not observed effects of
this magnitude at such low doses in recordings elsewhere in
the brain (especially the cerebellum).

This powerful effect on motor cortical unit activity could be
involved in some aspects of ethanol's disruption of motor
coordination. In addition, in the simplified model of this
circuit brought out in figure 2, such an action on motor
cortex could also bring about ethanol's observed disruption of
sensory gating in the somatosensory cortex. Figure 5
illustrates, in the same schematic format used in figure 2,
one possible explanation which is consistent with our findings
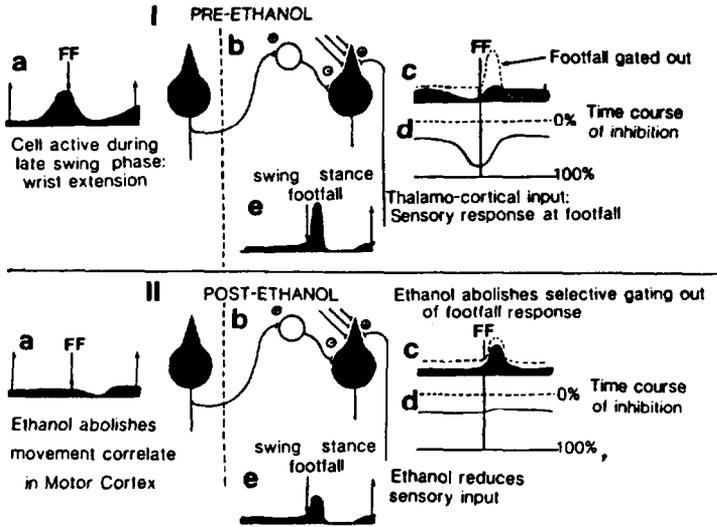
FIGURE 5

Schematic diagram showing hypothetical mechanism of ethanol's effect of sensory eating. "Pre-ethanol" schematic (top; I:a-e) is similar to that for type B cell in figure 2. I:e shows perievent histogram of hypothetical response to footfall in cutaneous afferent system; II:e shows response after ethanol. Normal type B pattern of cortical gating shown in I:c,d. Dotted line in I:c shows how perifootfall histogram would look in absence of gating; I:d shows perifootfall gating histogram. In the postethanol condition (II:c,d), there is some tonic inhibition, but no selective gating. Perievent histograms I:a and II:a show observed activity of motor cortical units over the step cycle in the pre- and post-ethanol conditions.

in both cortices. The "pre-ethanol" condition of a typical type B cell is illustrated at the top of figure 5. The "postethanol" condition (bottom) schematizes the observed reduction in the selective "gating-out" of the footfall response. This might be attributable, in this simple model, to a reduction in activity of motor cortical units which normally subserve such inhibition. While the real situation is undoubtably much more complex, this model appears consistent with all currently available evidence. If other regions of frontal or prefrontal cortex are similarly affected by such low ethanol doses, a wide range of perceptual, cognitive, and behavioral control deficits could result.

It is instructive to compare these actions of ethanol to the those of other sedating or anesthetic drugs, such as halothane,

barbiturate, and ketamine. We have found that each of these drugs produces a selective reduction in the longer latency (E1b) sensory responses of single somatosensory cortical neurons (Chapin et al. 1981a). However, the behavioral concomitants of the phenomenon vary considerably according to the drug. For example, a dose of ethanol capable of producing coma results in a virtual block of all somatosensory input to the cortex, whereas after barbiturate-induced coma, the short-latency response remains strong.

The "dissociative anesthetic" ketamine, on the other hand, produces markedly different effects than ethanol. First, it does not selectively reduce the longer latency component of the excitatory sensory response in the cortex, but instead reduces both early excitatory responses (E1a and E1b; see figure 3) and amplifies a much longer latency (>100 msec) component. Further, it does not disinhibit responses to sensory input during movement as does ethanol. Finally, whereas ethanol reduces both spontaneous and movement correlated discharge in the motor cortex, ketamine increases both categories of firing. Thus, while moderate doses of both drugs produce ataxic "staggering" behavior, ethanol is correlated with a reduction in motor and "associative" cortical activity, and ketamine is correlated with an increase in such activity. This is consistent with the results of Hammer and Herkenham (1983) who showed that metabolic activity (as indicated by uptake of 2-deoxyglucose) of associative regions of the rat cerebral cortex was markedly increased relative to primary sensory regions after ketamine administration.

DISCUSSION

The question to be considered is how results of experiments at the cellular level may relate to results of experiments at the systems level such as those described here. Our view is that the emergent properties of intact brain circuits make it difficult to account for drug-induced behavioral changes based on the results of information obtained from experiments at the cellular or membrane level. For example, in the awake, behaving animal we found that ethanol (and other sedative hypnotics such as barbiturate, or anesthetics such as halothane) markedly increased the postexcitatory inhibitory response to sensory stimulation. Initially, this appears to be consistent with a hypothesis suggested by Hunt (1983) that ethanol selectively enhances pre-synaptic release of gamma-aminobutyric acid (GABA).

On the other hand, our finding that the powerful inhibition of sensory input normally observed during movement was reduced, rather than increased, by ethanol seemed to contradict the notion that such phenomena are all attributable to increased GABA release. A possible explanation may lie in the suggestion (see figure 5) that this reduction in sensory

gating may be caused by a reduction in activity of motor cortex cells, which may normally provide the gating bias signal.

It is not clear at this point where or how ethanol acts to produce this effect on the motor cortex, and this is the crux of the matter. It is not unreasonable to suspect, for example, that frontal cortical excitability may be controlled by afferents rising through multisynaptic pathways from the basal ganglia, cerebellum, or brain stem, which themselves may be compromised by more direct physical effects of ethanol. This loss of excitatory input may decrease the ability of motor cortical neurons to respond appropriately during movement, thereby reducing the inhibitory input to SI.

Thus, the possibility that this drug effect may be caused by changes in the activity of neurons which may be several synapses removed from the recorded neuron reduces the predictive ability of a model based on information derived from reduced systems. At the very least, this reinforces the argument that an understanding of drug effects on behavior will require further knowledge not only of molecular, membrane, and synaptic mechanisms, but also of the circuit mechanisms. Overall, it will be necessary to build a continuity of understanding that serves to connect information gained at all these levels. The type of study described here is one way to address this issue.

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Correlating Behavior with Neural Activity: An Approach to Study the Action of Drugs in the Behaving Animal

Sam A. Deadwyler

INTRODUCTION

The assessment of drug action in the central nervous system (CNS) often requires three different but related approaches. A logical first step is the delineation of where in the brain or spinal cord the drug is likely to act. More specifically, it is necessary to have anatomical evidence that a given drug acts at a particular location in the nervous system. This type of evidence is supplied by the vast array of histochemical, biochemical, and more recently, molecular genetic techniques which provide information regarding the presence of receptors (Atweh and Kuhar 1977), endogenous ligands to bind to those receptors (Elde et al. 1976), and which neural structures incorporate those ligands (Cuello 1983; Sar et al. 1978; Walmsley et al. 1980).

The second approach is likely to be some assessment of the physiological action of the particular drug. Traditionally, examination of the neurophysiological effects of a drug in acutely anesthetized animals and observation of the neurological signs following injections in the unanesthetized animal have been the methods of choice. More recently, advances in brain slice technology (Dingledine 1984) have made it possible to study drug actions at the cellular and synaptic levels using intracellular and, most recently, single channel recording techniques (Sakman and Neher 1983). However, while slice electrophysiology-- which reveals the actions of drugs at single membrane loci and receptor elements--has supplied a great deal of new information regarding drug actions, there are obvious limitations in terms of extrapolating the findings to the intact nervous system.

A related approach to describing the functional consequences of drug action in the CNS has been to study the effects such influences might have on behavior. Psychopharmacological approaches have characterized various behavioral assays, and highly specific drug influences can now be detected and related closely to known pharmacological indices of drug action (such as rank-order potency, dissociation constants, PA2 analyses, etc. See Iverson et al. 1984). However, even in the latter case, once a drug-dependent behavior has *been* isolated and characterized, the linkage of the physiological substrate(s) to the behavioral changes produced by the drug are frequently little more than theoretical assumptions.

The third stage, therefore, in the complete analysis of the actions of abused drugs is to identify specifically those neural mechanisms altered by the drug which define the drug-dependent behavior. Historically, this has been the most difficult approach because it requires examination of neurophysiological mechanisms in behaving, unanesthetized animals. In the past, recording from freely moving animals required a high degree of technological sophistication. Now, however, the recording of concomitant neural activity and behavior has been perfected and is available to most neuroscience investigators. In addition, interpretation of such data has become more meaningful due to the use of identified cell populations with known I) neurotransmitter and neuromodular affiliations (cf. Jacobs et al. 1984; Foote et al. l983), or 2) identified inputs and outputs (cf. Evarts et al. 1971; Fetz and Finocchio 1979; Chapin et al., this volume).

OPIATE DRUGS IN THE BRAIN

Several important features of opioid action on neural tissue have been described in recent years (Bradley et al. 1978; Duggan and North 1984). Extensive investigations have shown that opiates and opioid peptides alter the physiological activity of nerve cells through specific receptor coupled membrane processes (cf. Miller 1984). The most commonly reported postsynaptic action of the alkaloid opiate compounds (morphine, levorphanol, etorphine, and normorphine) is a naloxone reversible depression in firing (Duggan and North 1984). The decreased firing results from an opioid receptor-mediated increase in potassium conductance. It is not yet clear whether this increase in potassium conductance is produced by an increase in intracellular calcium (North and Williams 1983b). It has become clear in recent years, however, that opiates probably have the same actions in many different cell types within the CNS. North and Williams (1983a) report that morphine and the opioid peptides leucine, methionine enkephalin, and appropriate analogs produce an increase in potassium conductance in the neurones of the locus coeruleus, substantia gelatinosa, and myenteric plexus. Although the consequences of this increased potassium conductance and subsequent decreased excitability may differ with respect to location and cell population within the CNS (Dingledine 1981; Nicoll et al. 1980), there is little evidence to suggest that opiates behave differently with respect to physiological actions on those cell types that possess opiate receptors. However, there are other effects of opiates on neural tissue that have been reported. These range from direct transmitter-like influences to humoral-like modulation of both pre- and post-synaptic mechanisms (Barker 1978; West and Miller 1983).

The discovery and subsequent confirmation by many investigators of multiple opiate receptors (Martin et al. 1976; Lord et al. 1977) have added a new dimension of complexity to the understanding of opioid action in central neural processes (cf. Martin 1984). In addition, a variety of opioid peptides have been described in the brain, spinal cord, and periphery (Imura et al. 1983; Hughes 1983). It is clear from these investigations that in the future a consideration of the specific nature of opioid action on central neural mechanisms will require a detailed analysis of the physiology and pharmacology of specific opiate receptor subtypes as well as a thorough investigation of their possible endogenous ligands (Watson et al. 1982; McGinty et al. 1984; Goodman et al. 1980; Atweh and Kuhar 1983). As has been recently reported (Walker et al. 1982; Dunwiddie et al. 1980). reversibility by the mu-receptor antagonist naloxone may no longer be a criterion for the unequivocal establishment of a neurophysiological effect mediated by opioid peptides.

EFFECTS OF OPIATE DRUGS IN HIPPOCAMPUS

One of the striking divergences from the consensus of reports regarding opiate and opioid peptide neural effects in the brain is the well-characterized excitatory influence of these substances in the hippocampus. Since Nicoll et al. (1977) initially reported the excitatory effects of both morphine and methionine enkephalin following microiontophoresis of these substances within hippocampus, there have been many detailed studies confirming the excitatory nature of opiates in this structure (cf. Corrigall 1983). The basis of the excitatory influence of opioids has been suggested to reside in the inhibitory action of opiates and opioid peptides on a select population of hippocampal inhibitory interneurones. The inhibition of these interneurones produces a disinhibition of pyramidal cells which receive tonic and/or phasic inputs from the opiate-sensitive inhibitory cells (Nicoll et al. 1980; Duggan and North 1984). This hypothesis was originally proposed by Zieglgansberger et al. (1979) on the basis of differential extracellular firing patterns from two distinct cell types following microiontophoresis of enkephalin in the CA1 region of hippocampus. Subsequent reports both in support of (Lee et al. 1980) and in conflict with (Haas and Ryall 1980; Dingledine 1981) the disinhibitory hypothesis have since appeared in the literature.

The analysis of the direct effects of opiate and opioid peptides on hippocampal cellular activity and synaptic mechanisms in CA1 has been provided by Dingledine (Dingledine 1981; Valentino and Dingledine 1982). These investigations have shown that mu- and delta-receptor agonists. but not kappa-receptor agonists, have dose-dependent effects on the synaptic and cellular excitability of pyramidal cells in the CA1 field of hippocampus. Concentrations of opiates (morphine) and opioid peptide analogs (D-ala-dleucine enkephalin) in the nanomolar range were shown to increase the excitability of hippocampal cells. These findings are consistent with the mechanism proposed recently by Newberry and Nicoll (1984) that opiates and opioid peptides decrease a dendritically located inhibitory influence which may be tonically or phasically activated by excitatory afferent terminals.

The results of in vivo experiments are consistent with the above in vitro hippocampal slice findings and have shown that apparent endogenous as well as exogenous opioid substances produce epileptiform discharges in hippocampus (Urca and Liebeskind 1979; Frenk et al. 1978, 1983; Henricksen et al. 1978). Behaviorally convulsant influences appear to be mediated by delta receptors, while epileptic discharges which do not produce behavioral convulsions are mediated specifically by mu receptors. Effective routes for producing the increased excitability of hippocampal tissue in vivo are intraperitoneal, intravenous, and intracerebroventricular (Frenk et al. 1983). The in vivo epileptiform effect, but not the convulsant aspects of opioid substances, can be reversed by systemic administration of naloxone.

In summary, it can be said that opiates with mu-receptor agonist properties and certain opioid peptides with delta-receptor agonist properties increase the excitability of hippocampal pyramidal cells. Kappa receptors are not implicated since opiate substances with high affinity for this receptor do not produce either epileptiform activity or convulsions at equivalent effective concentrations of mu- and delta-receptor agonists. The nature of hippocampal process activated by opioid receptor binding is not as yet identified. The most likely candidates for such a mechanism appear to be 1) an opiate receptor-mediated inhibition of the release of gamma-aminobutyric acid (GABA) from a specific population of

inhibitory interneurones (Zieglgansberger et al. 1979; Robinson and Deadwyler 1981; Nicoll et al. 1980). or 2) a phasically activated convergent inhibitory afferent fiber system with opioid receptors located on GABA axon terminals (Newberry and Nicoll 1984).
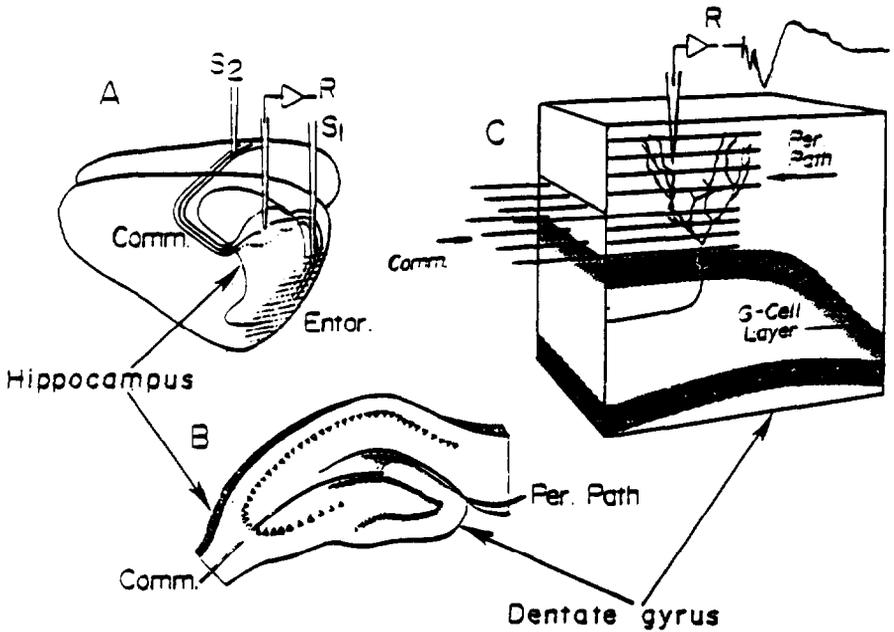
FUNCTIONAL SIGNIFICANCE OF OPIOID SUBSTANCES IN HIPPOCAMPUS

The presence of endogenous opioid peptides in hippocampus has been described in detail by Gall et al. (1981) and McGinty et al. (1983). These investigators have shown an enkephalin-like immunoreactivity in the perforant path region of the dentate gyrus and in the cells of origin of this pathway in the entorhinal cortex. These findings have importance for the eventual understanding of opioid peptide action in the hippocampus and possibly other limbic areas because of the considerable knowledge regarding the functional nature of this synaptic connection. The perforant path connection in the dentate gyrus exhibits several features which indicate a memory-like function (Lynch and Baudry 1984). In this context, the presence of an endogenous opioid peptide in the perforant pathway suggests a possible neurotransmitter or neuromodulator role of opiates in the establishment of long-term representations of sensory events. Haas and Ryall (1980) have demonstrated excitatory actions of enkephalin analogs on the granule cells of the dentate gyrus, the targets of the perforant path projections.

For several years, we have been developing a model of hippocampal function that has as its goal the identification of the behavioral circumstances which provoke sensory activation of synaptic pathways in the hippocampus of the unanesthetized, freely moving rat. In so doing. we have devised a means of studying the functional properties of the perforant path in the behaving rat (figure 1). Our past research has shown that an auditory evoked potential (AEP) recorded in the outer molecular layer (OM) of the dentate gyrus (the region of termination of the perforant path fibers) during performance of a sensory discrimination represents the transmission of sensory information into the dentate gyrus from the entorhinal and perirhinal cortex (Deadwyler et al. 1979a, 1979b, 1981). We have termed this evoked potential the OM AEP.

Prior investigations have documented several factors which control the amplitude changes in the two negative peaks (N1 and N2) (figure 2) of the OM AEP which appear to be specific to the stimulus context in which the animal performs the behavioral task. The Nl peak is large during acquisition of the behavior and decreases in amplitude as the tone stimulus gains significance through instrumental conditioning (figure 2). The N2 peak is nonexistent prior to conditioning and following extinction, but it increases and is maintained at maximal amplitude during acquisition and achievement of criterion behavioral performance. During extinction, the amplitude of Nl is increased and N2 is markedly decreased (figure 2). Recent evidence suggests that the N1 peak is a reflection of event-specific information regarding the nature of occurrence of positive-reinforced and negative-nonreinforced stimuli in a two-tone auditory discrimination task (West et al. 1982). Depth profile analyses of perforant path elicited extracellular field potentials and the N1 component of the OM AEP, as well as lesion studies which eliminated the entorhinal cortex or severed the perforant path connections to the hippocampus, strongly implicate the perforant path as the generator of the N1 component of the OM AEP (Deadwyler et al. 1981, 1982). The generator of N2 has not been identified; however, the integrity of the septohippocampal system is required for the behavioral modulation of this component (Deadwyler et al. 1981).

FIGURE 1

Recording procedures for chronic animal preparation. #A. Schematic drawing of entire rat brain showing orientation of stimulating (S1 and S2) and recording (R) electrodes. Stimulating electrodes are placed in the perforant path fibers (S1) originating from the entorhinal cortex (Entor. denoted by crosshatching in A) and the dentate gyrus commissural (Comm.) system (S2) originating in the contralateral hippocampus. The recording electrode (R) is placed in the dorsal hippocampus via phyiological criteria (i.e., electrically elicited monosynaptic field potentials) in each animal prior to behavioral testing. #B. An exploded drawing of the hippocampal region illustrated in A. A granule cell within the dentate gyrus is shown receiving convergent inputs from the two main fiber systems shown in A. These cells, in turn, project to hippocampal pyramidal cells, shown as triangles. #C. A further three dimensional expansion of this same dentate gyrus region showing a dentate granule cell with laminated afferent inputs from perforant path and commissural system with recording electrode positioned in the outer molecular layer (region of granule cell dendrites and afferent fiber systems) as during recording of the OM AEP. The perforant path region of the molecular layer is the location of enkephalinergic axons, as described by Gall et al. (1981).

The fact that the N1 component of the OM AEP seems to accurately reflect the perforant path synaptic input to the dentate gyrus indicates that it can be utilized as a "physiological marker" for the functional involvement of this pathway in behaviorally relevant neurophysiological processes. Because of our interests in the functional significance of the opioid peptides in the perforant path. we examined whether or not morphine and the potent mu-receptor agonist peptide FK 33-824 (Roemer et al. 1977) would alter the OM AEP in behaving rats (Christian et al., in press). Both intraperitoneal (5.0 to 7.0 mg/kg, morphine) and intracerebroventricular (36.0 ng/ul, FK 33-824) injections of these compounds increased the amplitude of the perforant path component (NI) of the OM AEP (figure 3). Intraperitoneal (i.p.) injections of naloxone (1.0 to 2.0 mg/kg) following morphine or FK 33-824 administration reversed the opioid-induced increase in the N1 and decrease in the N2 component (figure 3).

Examination of the extracellular synaptic current generated by monosynaptic electrical activation of perforant path fibers in these same animals showed naloxone reversible increases at all stimulus intensities, but were more marked at the higher voltages (figure 4). While these treatments did not have deleterious effects on behavioral responding at the indicated doses, increased doses (7.0 to 10.0 mg/kg) usually resulted in termination of behavioral responding even though the reinforcement contingency remained in effect.

Thus, opiates increased the N1 component of the OM AEP and the efficacy of the perforant path-to-dentate granule cell synapses simultaneously, while provoking an extinction-like correlate of the electrophysiological changes previously reported in nondrugged animals (see figure 2). In other studies, opioid substances have recently been shown to abolish both the behavior and the hippocampal neural correlate of the classically conditioned nictitating membrane response in the rabbit (Mauk et al. 1982. 1983). These findings suggest a function for the recently identified enkephalin-like opioid peptides within the perforant path and possibly other systems in the hippocampus. Whether or not these effects reflect a direct transmitter-type action of opiates and opioid peptides on dentate granule cells or modulation of other nonopioid transmitters in this region is not known. Gall et al. (1981) and McGinty et al. (1983) reported immunohistochemical labeling of opioid peptides associated with other regions of the dentate gyrus in addition to the perforant path. Enkephalin-like immunoreactivity was present in the mossy fiber axons and cell bodies of the dentate granule cells, which also showed positive reactivity for dynorphin (McGinty et al. 1983). The perforant path region is devoid of dynorphin immunoreactivity (McGinty et al. 1983). These results provide an anatomical basis for the above demonstrated electrophysiological effects of opioid substances on the OM AEP.

The results of our studies in the chronic animal demonstrate that exogenously administered opiates and opioid peptides alter the amplitudes of sensory evoked potentials in the hippocampus. A corresponding excitatory influence was obtained in recordings of monosynaptic field potentials elicited by electrical stimulation of the perforant path. Since the entorhinal cortex receives input from the primary cortical sensory areas (Van Hoesen and Pandya 1975), our results reveal a possible selective capacity for endogenous opioid peptides to modulate and/or regulate the transmission of sensory information into specific limbic brain regions (Lewis et al. 1981). Such a presumed action of endogenous opioid peptides would not be unlike that demonstrated in pain pathways in the
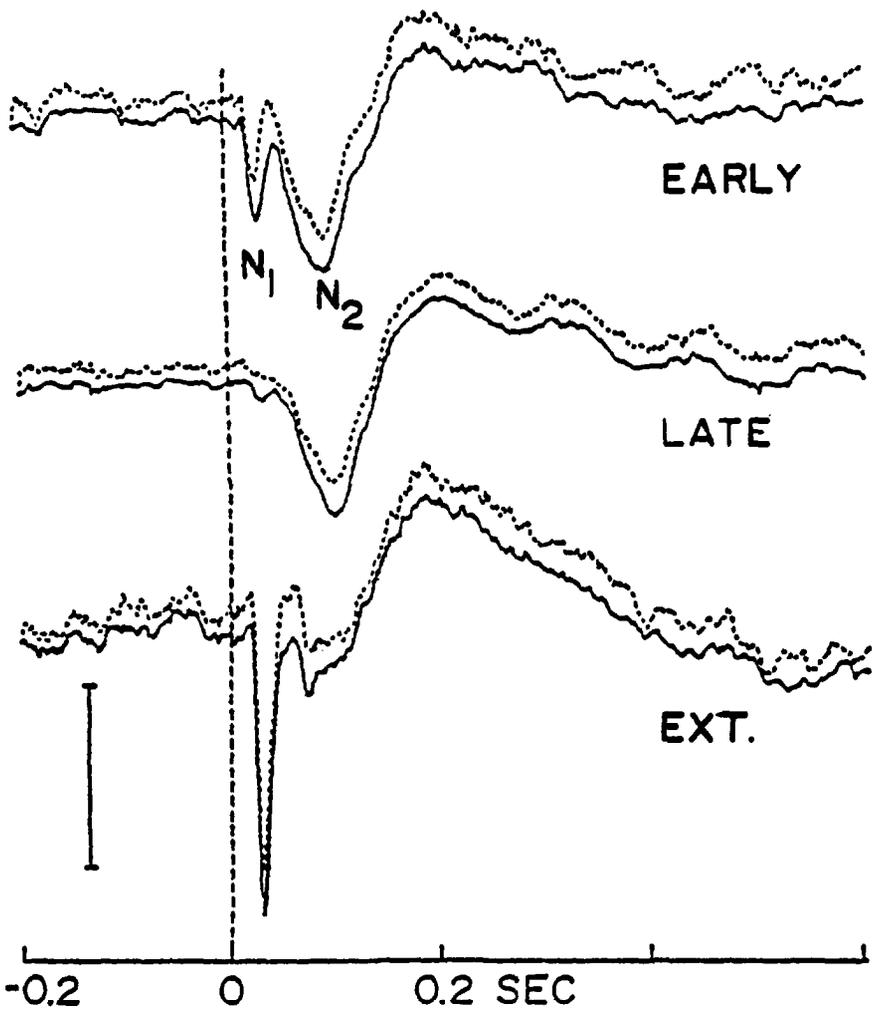
FIGURE 2

Examples of OM AEP's illustrating relevance to behavioral performance in auditory discrimintion task. Early: Top trace shows a composite (mean) of several OM AEPs (solid trace) recorded early in training prior to establishment of criterion performance. The N1 and N2 components are denoted underneath their respective peaks in this record. Each tracing is a composite normalized average of several individual OM AEPs recorded within different 50 trial training sessions. Dotted trace indicates session-to-session variance (standard deviation) of individual OM AEPs across 14 sessions. Late: Composite OM AEP recorded after achievement of criterion performance in the auditory discrimination task. Extinction: Lower trace illustrates changes in composite OM AEP during extinction of the auditory discrimination behavior. Calibration: 33 uV.

69
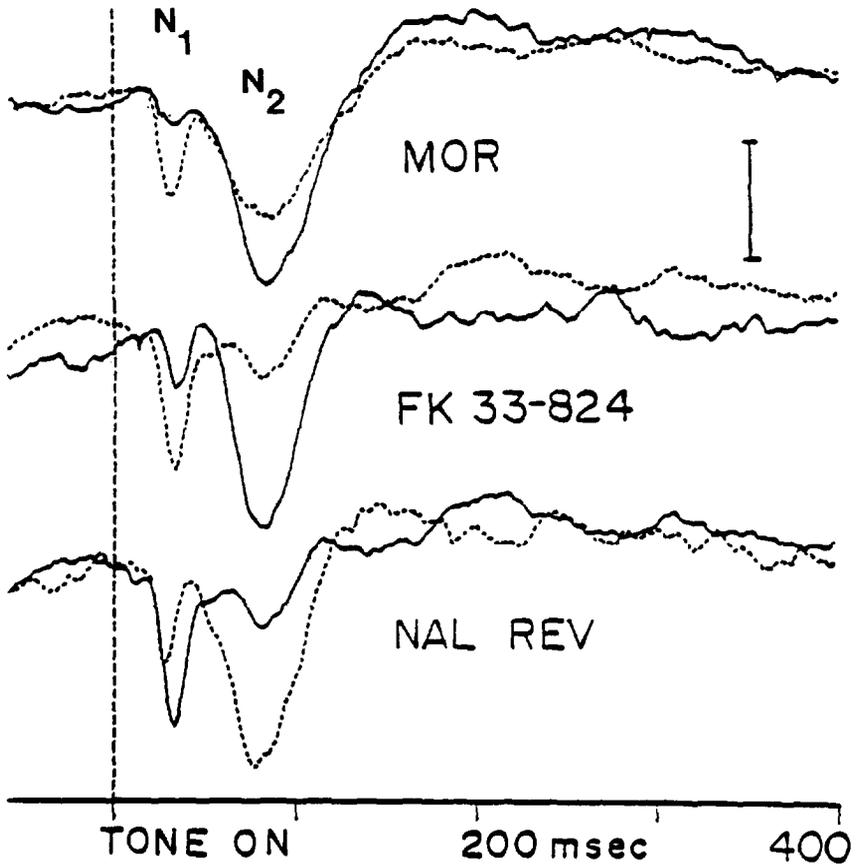
FIGURE 3

Effects of opioids on the OM AEP. Top trace (NOR): The effect of morphine administered i.p. on N1 and N2 components of the OM AEP. Solid curve is the control vehicle injection record. Dotted curve is the record obtained following a 5-7 mg/kg dose of morphine sulfate. Note increase in N1 and decrease in N2 caused by morphine. FK 33-824: Middle tracing illustrates similar but more dramatic effect of the mu-type receptor agonist FK 33-824 (Sandoz) administered intracerebroventricularly at a dose of 36 ng in 10 microliters of vehicle (saline). Large increase in N1 and reduction in N2 amplitude are similar to that seen with morphine (IP) and during extinction of the behavior (as illustrated in figure 2). NAL REV: Reversal of FK 33-824 effect (solid curve) by subsequent injection of 1-2 mg/kg of naloxone i.p. (dotted curve). Each trace is the normalized average of all sessions and all animals exposed to the indicated treatment.
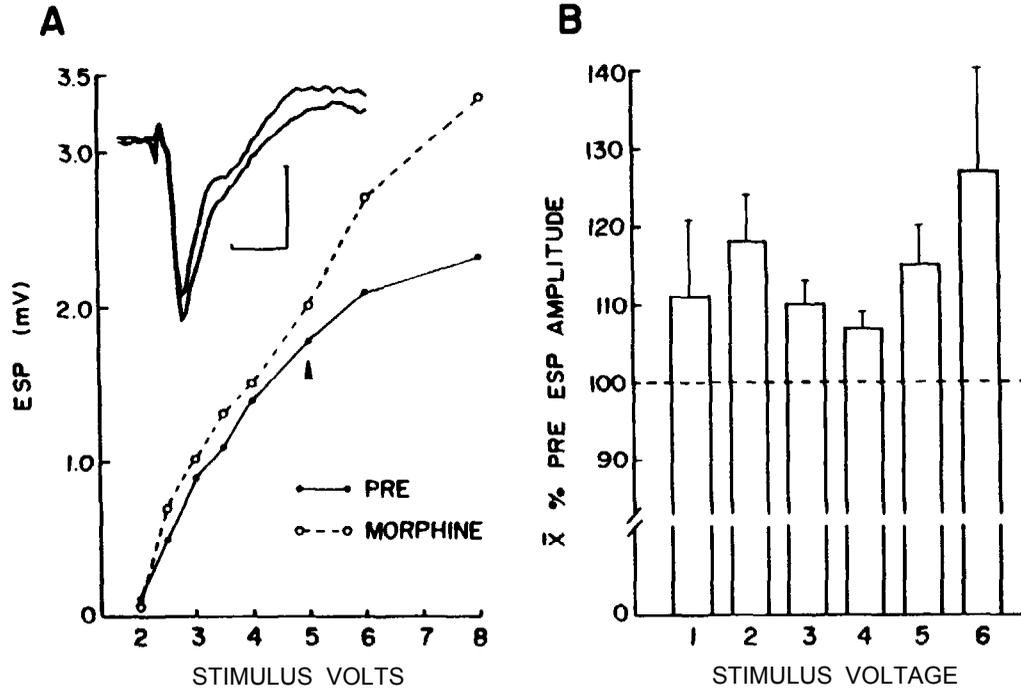
FIGURE 4

Effects of morphine on perforant path extracellular synaptic potential (ESP). Left: Input/output curve of ESP (see inset) prior to and after i.p. administration of 5.0 mg/kg dose of morphine. Calibration 1.0 mv and 5.0 msec. Arrow indicates voltages at which inset records were obtained on the curve. Right: Group data for three different animals showing percent change in ESP at different stimulus intensities following morphine injection (7 mg/kg). Bars indicate standard errors. Stimulus voltages were normalized across animals from lowest to highest intensities (100-400 uA).

spinal cord (Zieglgansberger 1980; Yaksh 1981; Cuello 1983). An opioid-regulated synaptic mechanism could, in this context, modify the responsiveness of hippocampal cells to sensory inputs arriving via the perforant path from the entorhinal cortex.

EFFECTS OF CANNABINOIDS IN HIPPOCAMPUS

Studies similar to those described above have recently been instituted to examine the psychoactive effects of cannabinoids on the OM AEP and unit discharges in the dentate gyrus. Animals trained in the same auditory discrimination task as described previously were subjected to low and high doses of delta-9-tetrahydrocannabinol (THC), the major psychoactive derivative of marijuana. These investigations showed that all doses of THC altered behavior in a dose-dependent manner. At doses between 0.5 mg/kg and 1.5 mg/kg there was a tendency to selectively increase responding (above control levels) to the negative (nonreinforced) tone in a two-tone discrimination task. These behavioral effects were not observed even with very high doses of opiates (>10.0 mg/kg). In contrast, i.p. doses of THC above 2.0 mg/kg caused the animals to cease responding altogether.

The above doses of delta-9-THC produced dose-dependent changes in OM AEPs. Control OM AEPs were stable across nondrug days. The 0.5 mg/kg dose slightly decreased N1 but not N2 in the OM AEP, however, doses of 1.0 to 2.0 mg/kg produced a substantial decrease in N1 recorded during both single and differential tone discrimination performance. N2 amplitude was increased when N1 was decreased and vice versa. Like opiates, these changes were observed for up to 2 hours after drug administration, at which point they tended to recover to control levels.

The systematic effects of dosage indicate that the changes in the OM AEP may be functionally related to corresponding changes in the behavior observed at the same dose levels. The data indicate that 1) dentate gyrus neural activity is affected by pharmacological doses of delta-9-THC, and 2) dose-dependent changes can be expected from usage of this substance. The above findings confirm the fact that the effective dose range for observing alterations in hippocampal activity in behaving rats is similar to the dose range reported previously for other, unanesthetized preparations (Turkanis and Karler 1981). The biphasic action of this substance previously documented in in vitro preparations (Vardaris et al. 1977; Weisz et al. 1982), however, was not observed in this study. Furthermore, our results suggest that gross disruptions in behavioral performance probably occur within the same dose ranges in which hippocampal effects were observed. It has recently been shown that cannabinoids exert a profound effect on memory in humans and it has been suggested that the effects are mediated via alteration in the cholinergic projections to the hippocampus (Miller and Branconnier 1983; Cheney et al. 1981). Our results are compatible with these assumptions.

CONCLUSION

The arsenal of techniques available to examine the effects of drugs of abuse on neural processes now encompasses recordings made at the single cell and subcellular levels, as well as the detection of changes in neural events related to "associative" or "cognitive" processes in behaving animals. These techniques, coupled with equally sophisticated biochemical and anatomical analyses of drug

dispersion, site of action, and relation to endogenous neurotransmitter and neuropeptide systems, make it possible to characterize the continuum of neural effects necessary for determining the abuse potential of untested drugs, while providing new insights into the neural effects of more traditionally abused substances. The use of chronic animal preparations will allow determination of not only the consequences of drug action on behavior but also the effects on identified neural events following acute, as well as repeated, drug exposure.

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The Use of Event-Related Potentials In the Study of Alcoholism: Implications for the Study of Drugs of Abuse

B. Porjesz and H. Begleiter

While it has long been apparent that the human brain is susceptible to both the acute and chronic effects of alcohol, it is only recently, with the development of sophisticated computer-assisted technology, that these effects can be studied in vivo. With the use of this technology, evoked brain potentials (EPs) can be studied to examine more subtle forms of brain damage and/or dysfunction by examining the relation of an EP to a specific sensory or behavioral event. Event-related potentials (ERPs) offer a unique approach for assessing level of brain functioning, as they permit the simultaneous observation of electrophysiology and cognition.

An ERP is obtained by recording with a non-invasive scalp electrode the time-locked brain electrical activity that follows the delivery of a discrete stimulus to any sensory modality (e.g., auditory, visual). Signal averaging techniques make it possible to extract these time-locked neuroelectric signals (i.e., the ERPs) from the background random "noise," which is canceled out with these procedures. Depending on stimulation parameters and recording sites, these time-locked signals represent activity at neural generators from the peripheral end organ to higher integrative centers of the brain. The quantitative measurement of salient features extracted from ERP recordings provide objective neurophysiological data reflecting various aspects of brain function and integrity.

ERP techniques have proven to be especially valuable in indexing electrophysiological concomitants of complex cognitive tasks (Hillyard et al. 1978; Donchin 1979; Donchin et al. 1978). They can be recorded in conjunction with behavior, or even when no behavioral response is required, to both attended and unattended stimuli. Thus, the ERP techniques are very sensitive indices of the functional integrity of the brain. They differ from computerized axial tomography (CT-scan) or nuclear magnetic resonance (NMR) in that they reflect subtle, dynamic, moment-to-moment changes in brain functioning that are elicited

while the brain is being challenged, rather than the static gross brain damage that is apparent on the scans. As a result, ERP aberrations are often observed in the absence of brain damage as visualized on CT-scan.

In order to consider how EP techniques may be used to study problems of drug abuse, it may be useful to examine how they have been used to study the effects of alcohol on the brain. EP techniques have provided indices sensitive to the various alcohol effects, namely: alcoholization, tolerance, withdrawal, and long-term brain dysfunction resulting from chronic alcohol use. Alcoholization is characterized by marked decreases in EP amplitude (Bierley et al. 1980) and prolongations in conduction velocities of the brain stem potential (BSP) (Squires et al. 1978a, 1978b; Chu et al. 1978). Chronic alcohol intake is also accompanied by EP amplitude reductions (Porjesz et al. 1976; Begleiter and Porjesz 1977) and by BSP delays that are less pronounced once tolerance develops Squires et al. 1978a, 1978b; Chu et al. 1978; Zilm et al. 1981). Withdrawal is characterized by increased EP voltages and extremely shortened BSP latencies, suggestive of underlying CNS hyperexcitability (Begleiter and Porjesz 1977, 1979; Begleiter et al. 1980a; Squires et al. 1978a, 1978b; Chu et al. 1978; Hunter and Walker 1980). Finally, long-term abstinence is marked by decreased EP amplitudes (hyporeactivityj and prolonged BSP latencies and slower conduction velocities (Begleiter et al. 1981; Porjesz and Begleiter 1983). The duration of these prolonged central nervous system (CNS) disturbances and their potential recovery are not yet known.

We have recently recorded auditory BSP's from hospitalized alcoholics who were abstinent from alcohol for 1 month (Begleiter et al. 1981). This technique allows investigation of subcortical brain functioning with a noninvasive scalp electrode (Sohmer and Feinmesser 1967; Jewett 1970; Jewett and Williston 1971). These "far-field" potentials consist of 7 time-locked positive waves, (designated I to VII), each presumed to reflect activity at different sites along the auditory pathway from the auditory nerve through the brain stem (Jewett 1970; Lev and Sohmer 1972; Buchwald and Huang 1975; Starr and Achor 1975; Starr and Hamilton 1976; Stockard and Rossiter 1977). The latencies of each of these peaks, as well as "central conduction time" (the latency of each peak with respect to peak I), are accurate in localizing sites of pathology from the peripheral end organ to the brain stem; the time interval between the first peak and peak V of the inferior colliculus is most often taken as a measure of brain stem transmission time (Fabiani et al. 1979).

In our study, we found that alcoholic patients manifested significant delays in latencies and central conduction velocities of peaks II to V. These findings are remarkably similar to those reported with acute doses of alcohol in animals (Squires et al. 1978a) and man (Squires et al. 1978b). Our study provided the

first systematic electrophysiological evidence of brain dysfunction at levels other than neocortex in alcoholics without overt clinical signs of neurological damage. The increase in neural transmission time may reflect the process of demyelination, which has long been suspected in alcoholics (Adams et al. 1959) and has been observed in rats chronically exposed to alcohol (Moscatelli and Demediuk 1980). Similar results have recently been reported in neurologically impaired alcoholics (Rosenhamer and Silfverskiold 1980; Chu and Squires 1980; Nickel and Ludewig 1981; Haas and Nickel 1981; Chu et al. 1982), and in neurologically intact alcoholics (Cassvan et al. 1984). The etiology of these auditory BSP delays and the drinking history factor(s) (e.g., length of drinking history, amount consumed per sitting, number of withdrawals, and nutritional factors) that result in brain stem aberrations have not yet been definitively determined. It is even possible that nutritional deficiencies alone produce demyelination and hence the BSP delays because nutritional deficits are known to lead to demyelinating diseases such as polyneuropathy (Hillman 1974). At present, we are investigating the relationship between drinking history, nutritional factors, and the magnitude of BSP aberration. Our preliminary data suggest that alcoholics with signs of nutritional deficits and/or polyneuropathy display different BSP waveforms than those alcoholics without nutritional deficits. Furthermore, length of drinking history does not seem to correlate with BSP delay; in fact, alcoholics with relatively short heavy drinking histories (< 8 years) and evidence of nutritional deficits manifested greater BSP aberrations than alcoholics with long drinking histories (> 20 years) and no signs of nutritional deficits. The results of animal studies (Chu et al. 1978) suggest that other factors besides chronic alcohol exposure are necessary to produce BSP abnormalities; chronic alcohol ingestion was not sufficient to cause BSP delays after withdrawal in laboratory animals that did not also have nutritional deficits. Taken together, these findings suggest that BSP aberrations in alcoholics may be the result of alcohol and/or nutritional factors.

For the past several years, we have also systematically examined ERPs in medication-free alcoholics who are abstinent for approximately 1 month. These ERP techniques require the subject to be engaged in a task (usually information processing). Each task is designed to examine deficits of a particular ERP component which has been well documented to vary predictably under specific conditions in normal subjects (Hillyard et al. 1978; Donchin et al. 1978).

In one bimodal (visual and auditory) study, we investigated the ability of alcoholics to focus on a relevant stimulus modality and inhibit responding to an irrelevant modality by examining the Nl component of the ERP (Porjesz and Begleiter 1979), which occurs at around 100 msec. The Nl component is sensitive to attention to a relevant stimulus modality; it is enhanced to

all stimuli in a relevant stimulus modality, and reduced to stimuli in irrelevant modalities (Hillyard et al. 1973, 1978; Picton and Hillyard 1974). The patient was presented with a sequence of randomized single flashes and clicks with rarely occurring double flashes and clicks interspersed among them. The patient was required to "shift attentional sets," in order to count either the double flashes or double clicks, or ignore all stimuli, in an otherwise identical stimulus sequence.

Consistent with the ERP literature (Hillyard et al. 1973, 1978), control subjects in our study manifested significantly enhanced Nl components to stimuli in the relevant as compared to the irrelevant modality; however, alcoholics maintained the same low amplitude of N1 regardless of the degree of task relevance. Often it is the differential voltage between ERPs recorded to stimuli in relevant and irrelevant channels that is more revealing about the nature of brain functioning than absolute voltages to either relevant or irrelevant stimuli. These results suggest that alcoholics may be incapable of appropriate "sensory filtering," as they do not differentiate neurophysiologically between relevant and irrelevant channels.

In addition, the results indicated that abstinent alcoholics manifested abnormally reduced late component amplitudes ($\geq$ 100 msec), but not early component amplitudes. These findings in abstinent alcoholics are remarkably similar to results obtained when healthy subjects ingest single doses of alcohol (Lewis et al. 1969; Porjesz and Begleiter 1975; Rhodes et al. 1975). This suggests that the neurophysiological brain dysfunction observed in abstinent alcoholics may resemble brain functioning detected in normal persons under the influence of alcohol.

We have also used the P3 component of the ERP in many different experimental paradigms to examine brain dysfunction in alcoholics (figure 1). The P3 or P300 component is a large, positive deflection that occurs approximately 300 msec to 500 msec after the stimulus. It can only be elicited under certain rather specific conditions related to the "subjective significance" of a stimulus, namely: task relevance (Sutton et al. 1967), unpredictability (Donchin et al. 1978) and infrequency (Tueting et al. 1971). as well as by motivational factors (Begleiter et al. 1983). The characteristics of P3 are unrelated to stimulus parameters, and can even be elicited in the absence of an expected stimulus (emitted potentials) (Klinke et al. 1968). In terms of scalp topography, P3 has been found to be maximum over parietal areas; it is bilaterally distributed without apparent hemispheric asymmetry, with similar distributions regardless of the sensory modality of the stimulus (Simson et al. 1976, 1977a, 1977b).

In one of our laboratory studies, we investigated the P3 component with a visual target-selection paradigm (Porjesz et al. 1980). The target-selection paradigm is most frequently used to
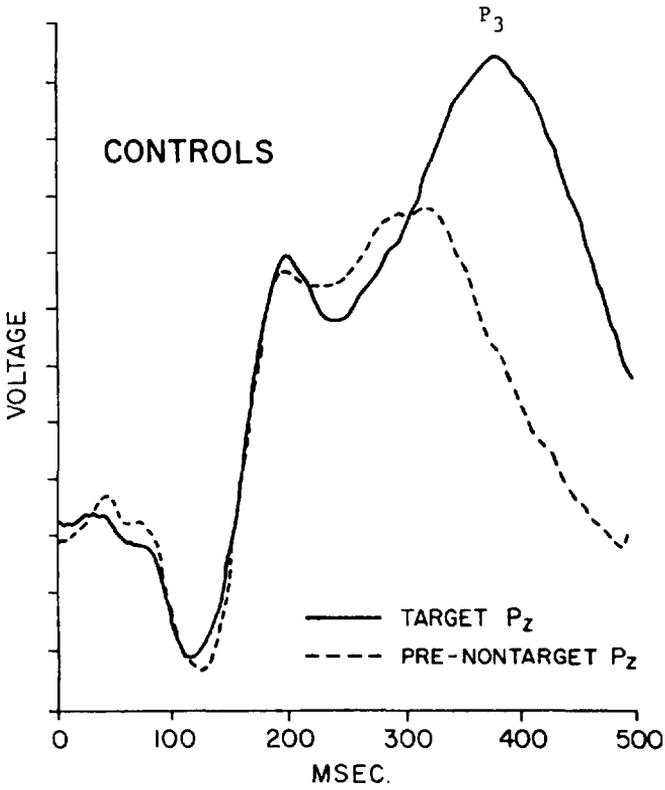
FIGURE 1

Grand mean ERP wave forms recorded at parietal electrode (Pz) to the target stimulus (solid linej and nontarget stimulus (dashed line) in healthy subjects. Notice the prominent P3 component (large positive deflection occurring between 300 and 450 msec) to the target stimulus.

elicit a P3 component; it requires the subject to detect a designated rarely occurring target stimulus embedded in a series of frequently occurring nontarget stimuli. ERPs recorded to frequently occurring nontarget stimuli elicit N1 components, but no P3, while rare target stimuli elicit both N1 and P3 components (figure 1). In our study (Porjesz et al. 1980), the stimuli were geometric shapes. One rare geometric shape (e.g., triangle) was designated target, and the subject was required to press a button only in response to that stimulus. Target and nontarget stimuli were alternated every other block enabling the recording of ERPs to the same stimulus when it was target or nontarget. ERPs were recorded to targets (rarely occurring, task-relevant geometric shapes), nontargets (frequently occurring task irrelevant geometric shapes), and novel stimuli (rarely occurring task irrelevant random shapes).

We found that P3 amplitudes were significantly reduced or absent in alcoholic patients to rare target stimuli under conditions optimal for eliciting large P3s (Donchin et al. 1978) (figure 2). This finding was most pronounced over parietal areas, where P3 amplitude is maximal at scalp (Simson et al. 1577a. 1977b; Ritter et al. 1968). Furthermore, while controls manifested differentially enhanced P3 components to target stimuli, alcoholics manifested identical low amplitude P3 waves with the same P3 latencies, regardless of whether a stimulus was a target or nontarget. Moreover, despite the fact that all stimuli were in the relevant channel, we found that the N1 amplitudes were significantly reduced in alcoholics to all stimuli, to levels comparable to an irrelevant stimulus modality. As in our bimodal study (Porjesz and Begleiter 1979), this suggests that sensory-filtering mechanisms are impaired in chronic alcoholics.

Thus, the major ERP aberrations manifested by alcoholics are the lack of differentiation between their responses to relevant and irrelevant inputs, and the low voltages of their event-related activity. This seems to suggest underlying brain dysfunction that impairs sensory-filtering and "probability-matching" processes.

Recent evidence implicates the amygdala and hippocampus as possible neural yenerators of P3. One recent study investigating the neural origins of P3 with implanted electrodes in humans reported that P3 was maximum at subcortical loci (Wood et al. 1980). Similarly, Halgren et al. (1980) have recently recorded large late potentials from limbic system with implanted electrodes in humans. They postulate that P3 may be generated in hippocampus or amygdala. Magnetoencephalographic studies have also suggested the hippocampus as a possible neural generator of the P3 component (Okada et al. 1983).

Thus, our results that alcoholics manifest low-voltage or even absent P3 components under conditions designed to elicit maximum P3 component amplitudes may be indicative of hippocampal

ALCOHOLICS

VOLTAGE

TARGET P$_z$

PRE-NONTARGET P$_z$

0   100   200   300   400   500

MSEC.

FIGURE 2

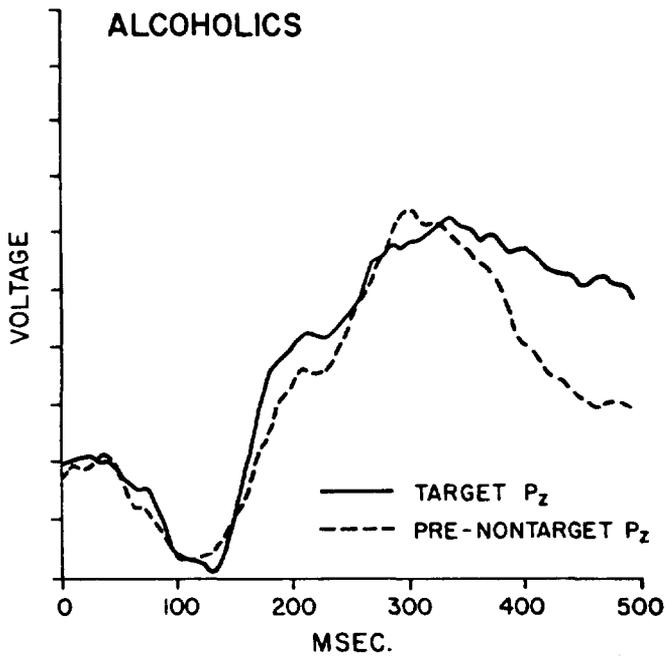Grand mean ERP wave forms recordea at parietal (Pz) to the target
(solid line) and nontarget (dashed line) stimuli in the alcoholic
group. Compare the P3 component of the target stimulus to that
of the control group (figure 1). and notice how reduced it is in
amplitude. Also notice the lack of difference between P3
amplitudes to target and nontarget stimuli in the alcoholic group
in this figure.

deficits. While these results do not rule out the contributions of neocortical sites, they emphasize the important role of limbic structures in generating the P3 component. The involvement of the hippocampus in chronic alcohol intake in the absence of malnutrition has been recently demonstrated in neuropathological and neurophysiological studies in animals (Begleiter et al. 1980a; Walker et al. 1980, 1981; Riley and Walker et al. 1978). Long-term ethanol consumption has been found to result in the loss of dendritic spines in the hippocampus of both the mouse (Riley and Walker 1978) and rat (Walker et al. 1980). In our laboratory, we have also demonstrated a hippocampal susceptibility to both acute and chronic alcohol effects with EPs recorded from monkey hippocampus (Begleiter et al. 1980a).

We were interested in determining the relationship between electrophysiological deficits and structural deficits assessed with CT-scan in alcoholics (Begleiter et al. 1980b). We selected two groups of alcoholics who had been subjected to CT-scans following 1 month of abstinence: namely, those manifesting a high degree of widened cortical sulci (Pos-CT) and those without any evidence of widened cortical sulci (Neg-CT). Patients in the two groups did not differ with regard to age, education, and drinking history (duration and amount). ERPs were recorded on the same day as the CT-scan and involved the same P3 paradigm previously described (Porjesz et al. 1980). Alcoholics with enlarged cortical sulci (Pos-CT) had significantly reduced (or absent) P3 amplitudes to target stimuli, as compared to alcoholics without signs of enlarged cortical sulci (Neg-CT); however, both groups manifested smaller P3s to targets than did control subjects. Furthermore, both groups of alcoholics displayed similar P3 components to all categories of stimuli, regardless of task relevance.

Because Neg-CT alcoholics also manifested diminished P3 amplitudes when compared to healthy nonalcoholics, neocortical shrinkage alone cannot explain these P3 reductions. These findings suggest that chronic alcohol abuse not only results in changes in the neocortex, but may also involve electrophysiological aberrations indicative of other brain (e.g., limbic system) deficits. Often the neocortical deficits in chronic alcoholics are emphasized while subcortical aberrations are overlooked. Our results suggest that alcoholics manifesting observable widened cortical sulci on CT-scan are more likely to also manifest hippocampal deficits. This, perhaps, lends support to the hypothesis that alcohol produces diffuse brain damage not solely circumscribed to neocortical areas.

We have recently completed a study examining the N2 or N200 component of the ERP in abstinent alcoholics (Porjesz and Begleiter 1981a, 1981b; Porjesz and Begleiter, in press). The N2 component is a modality-specific negative deflection with a maximum amplitude at occipitoparietal scalp for the visual modality and at central regions for the auditory modality.

Recent evidence suggests that the latency of N2 can be taken as an early index of stimulus evaluation time (Renault and Lesevre 1979); the more difficult the discrimination, the longer the latency of N2 (Ritter et al. 1979; Towey et al. 1980; Gaillard and Lawson 1980). This component is thus a better index of stimulus evaluation time than the reaction time (RT), because it is not confounded by the motor response. The RT is a complex measure of speed of information processing, as it depends on the end product of stimulus evaluation, response selection and organization, and motor response. Therefore, although there are some reports of delayed RTs in chronic alcoholics (Talland 1963; Vivian et al. 1973; Bertera and Parsons 1973), these studies cannot determine which aspect(s) of information processing is (are) slower in alcoholics.

In order to use the N2 component of the ERP as an index of speed of stimulus evaluation, we designed an RT study involving easy and difficult line orientation discriminations. This visuospatial RT design enabled us to investigate the relationship between difficulty of discrimination, N2 latency, P3 characteristics, and RT in abstinent alcoholics. ERPs were obtained to frequent nontarget (vertical line), and infrequent easy (90 degree deviant from vertical) and difficult (3 degree deviant) line orientations.

Our results indicated that the latency of N2 reflected difficulty of discrimination in the control subjects, being significantly delayed to the difficult when compared to the easy discrimination. By contrast, it failed to do so in the alcoholics, where N2 latencies were similar regardless of difficulty of discrimination. Furthermore, the N2 latency occurred significantly later in the alcoholic group than in the control group for both easy and difficult discriminations, suggesting that alcoholics need more time for stimulus evaluation and may therefore find the discrimination task more difficult. The latency difference between groups was even more apparent for the easy discrimination than for the difficult discrimination. This suggests that alcoholics need proportionately more time to make an easy discrimination (vertical from horizontal) when compared to controls (who can process this information more quickly), than to make a difficult discrimination (which both groups presumably find difficult).

In addition, alcoholics manifested delayed P3 latencies to easy discriminations when compared to controls; these P3 latencies were comparable to those expected for a difficult task. These results suggest that alcoholics adopt an undifferentiated mode of responding regardless of task requirements, finding all tasks difficult. While the amplitude of N2 was larger for easy discriminations than difficult discriminations in the control group, the amplitude of N2 was the same in the alcoholics regardless of task difficulty. The amplitude of N2 has been

shown to be related to the degree of stimulus deviance in normal subjects (Naatanen 1981).

There were no significant differences in RTs between the two groups of subjects, although the alcoholics tended to have faster RTs and make more errors than controls. This response pattern implies that alcoholics adopt different response strategies from controls, stressing speed over. accuracy (Kutas et al. 1977). Their apparent inability to withhold responding until certainty of accuracy or correctness has been established suggests a lack of inhibition in alcoholics.

In addition to these latency results, we found that alcoholics had significantly decreased P3 amplitudes. This low amplitude P3 was even more apparent for the easy discrimination, where controls exhibited very high P3 voltages. The P3 voltage was significantly larger for the 90 degree target when compared to the 3 degree target in the control but not the alcoholic subjects. This result is predicted by many ERP studies which have demonstrated that the more deviant a rare stimulus is from the background (the more easily discriminable it is) the larger the P3 amplitude (Towey et al. 1986; Ritter et al. 1972; Ford et al. 1979; Johnson and Donchin 1978; Ruchkin and Sutton 1978). Perhaps the lack of P3 amplitude difference in the alcoholic group indicates that they are more uncertain of the correctness of their decision than are controls, as they stress speed over accuracy.

Thus, on the basis of both the N2 and P3 ERP components, it was concluded that alcoholics have difficulty evaluating the potential significance of a stimulus. They do not electrophysiologically differentiate between relevant and irrelevant or easy and difficult discriminations, but rather maintain the same ERP characteristics (both amplitude and latency) regardless of the task requirements. This perhaps indicates that their template for match/mismatch decisions is lost or not readily available. In either case, it suggests a memory deficit where each incoming stimulus must be evaluated anew. Our data suggest that alcoholics manifest both types of brain deficits: the delay in N2 latency suggests that the template for comparison is not as readily accessible in alcoholics, while the low P3 voltages suggest that once retrieved, the match/mismatch processes themselves are impaired in alcoholics.

We are currently examining the reversibility of the BSP and ERP deficits observed at 1 month of abstinence and following 4 months of continued abstinence in the same hospitalized alcoholics (Porjesz 1983; Porjesz and Begleiter, in preparation). Preliminary data following 4 months of abstinence indicate improved morphology of BSP waveforms, shortening of latencies, and improved conduction times.

The relative roles of abstinence from alcohol and nutritional factors in so-called "recovery" still remain to be determined. Throughout the long-term abstinence program in our hospital, patients receive extensive vitamin therapy and may be manifesting improvements in nutritional status. Furthermore, the role of withdrawal cannot be overlooked; CNS hyperexcitability may be followed by a period of subacute hypoexcitability. We might speculate that this hypoexcitability is manifested by a prolongation of brain stem latencies caused by aberrant fluidizing effects on the neuronal membranes which may result in edema. Edema resulting from osmotic stress can lead to demyelination (Lewis 1976; Yates 1976; Feigen and Budzilovich 1978, 1980; Kleinschmidt-DeMasters and Norenberg 1981).

However, it should be noted that those alcoholics who remained in treatment for the full 4 months had less impaired BSPs at initial testing (3 to 4 weeks) when the data were analyzed retrospectively. As we are only able to examine reversibility in alcoholics who remained in long-term treatment, and these alcoholics tend to be less impaired initially, we cannot be certain that recovery occurs in all alcoholics regardless of degree of impairment. It remains to be determined whether recovery occurs as a function of the initial degree of impairment, whether greater impairment requires longer time periods for reversibility, or whether recovery ceases beyond a certain critical level of impairment.

We are currently investigating electrophysiological aberrations in another group of nonhospitalized alcoholics sober from 3 to 10 years, and thus far we have found that they manifest normal BSPs. This suggests that perhaps 4 months is not a long enough time interval to investigate reversibility of brain dysfunction following years of heavy drinking. However, it should be noted that these long-term abstinent alcoholics (> 3 years) were not tested initially; therefore the extent of BSP aberration they may have manifested immediately following alcohol abuse is not known. It is possible that these alcoholics never exhibited BSP delays, as we do not see BSP delays in all alcoholics tested. While the issue of reversibility is still unresolved, the data seem to indicate slow reversibility of BSP deficits with prolonged abstinence.

Although the BSP delays seem to improve with prolonged abstinence, the decreased voltages in the P3 component of the ERP do not seem to change with prolonged abstinence (Porjesz 1983; Porjesz and Begleiter, in preparation). We examined the possibility of reversibility of late component P3 deficits in abstinent alcoholics following 3 weeks and 4 months of abstinence. Interestingly, no improvement in ERP morphology or late component amplitude was noted following 4 months of abstinence in the same alcoholics; in fact, the waveforms were strikingly similar at initial test and retest. Furthermore, there was no improvement in the differential enhancement of P3

amplitudes on the basis of task relevance to target stimuli in
these abstinent alcoholics. In addition, P3 aeficits were still
observed in our group of nonhospitalized alcoholics sober from
3 to 10 years. Thus, even following 3 to 10 years of abstinence,
alcoholics still manifest abnormally low P3 amplitudes. We have
observed these P3 decrements in response to both auditory and
visual target stimuli in a bimodal target-selection study. These
results suggest that the P3 deficits may not be reversible, or
perhaps reverse much more slowly following very long abstinence
periods.

Thus, it seems that some electrophysiological aberrations
observed in alcoholics improve with prolonged abstinence while
other electrophysiological aberrations do not change with
prolonged sobriety. Caution is suggested in interpreting the
results as they are based on small samples. We are currently
examining this issue with larger sample sizes in an effort to
determine the important factors of susceptibility and
reversibility of brain dysfunction in alcoholism.

As these P3 deficits do not seem to recover following prolonged
sobriety (> 3 years), it is even possible that these deficits
may precede the development of alcoholism. While it has
generally been assumed that the brain abnormalities observed in
alcoholics are due to the toxic effects of alcohol on the brain,
nutritional deficits, or an interaction of alcohol and
nutritional-related factors, these brain deficits may represent a
predisposing factor differentiating those individuals with a
susceptibility to alcoholism. There is increasing evidence that
certain individuals are at high risk for developing alcoholism.
Specifically, sons of alcoholic fathers are four times more
likely to develop alcoholism than sons of nonalcoholics (Goodwin
1979; Goodwin et al. 1973), even when they are separated from
their biological parents soon after birth. Studies of male
adoptees indicate that the biological rather than the adoptive
parent is predictive of later drinking problems (Goodwin et al.
1973; Bohman 1978; Cadoret and Gath 1978; Cadoret et al. 1980;
Goodwin and Guze 1974; Schuckit et al. 1972). Furthermore, the
concordance rate for alcohol abuse among identical twins is
almost double the rate for fraternal twins (Kaij 1960); patterns
of alcohol consumption have also been found to be highly
concordant among identical twins (Partanen et al. 1966; Jonsson
and Nilsson 1968; Loehlin 1972). Taken together, these studies
suggest that a genetic factor predisposing sons of alcoholics to
alcoholism may be involved.

An exciting use of EP techniques is in identifying possible
biological marker(s) for those at risk for developing alcoholism.
It is likely that brain function is involved in the genetic
predisposition for alcoholism. There is good evidence to
indicate that brain EP waveforms are genetically determined.
Monozygotic twins, for example, manifest EP waveforms that are as
concordant with each other as EPs obtained from the same

indiviaual tested twice (Dustman and Beck 1965).

he have recently undertaken a major project which has already
demonstrated the possibility that sons of male alcoholics
manifest differences in EP that antedate any exposure to alcohol.
In order to study this problem, we are recording ERPs in boys
between the ages of 6 to 18 and comparing electrophysiological
responses from sons of alcoholics (high risk) and from age and
education-matched sons of nonalcoholics (low risk). Children
whose mothers abused alcohol are excluded to rule out the
contribution of the fetal alcohol syndrome (FAS).

In one ERP study (Begleiter et al. 1984), we examined the P3
component in boys between the ages of 7 to 13 who had no prior
experience with alcohol. The high-risk (HR) group consisted of
25 sons of alcoholic fathers with a mean age of 11.9 (S.D. =
2.1). In each case, the father had received the exclusive
diagnosis of alcoholism (DSM III) and had been in treatment for
alcoholism at some time. Boys whose mothers had ingested alcohol
during pregnancy or who drank excessively after giving birth were
excluded. Only boys without medical problems and without
exposure to alcohol or other substances of abuse were included in
this study. The 25 normal control subjects (NC) were boys who
were matched for socioeconomic status (SES) and age to the HR
subjects. The NC group had a mean age of 12.5 years (S.D. =
2.4). They were included only if they had no exposure to alcohol
or other substances of abuse, and had no history of alcoholism or
other psychiatric disorder in first or second degree relatives.
Except for alcohol history, the same exclusion criteria were used
as for the HR group.

The experimental design consisted of a visual head orientation
task (figure 3). Subjects pressed one of two microswitches as
quickly and accurately as possible (reaction time) with either
the right or left index finger to indicate whether the right or
left ear was present on the display, respectively. They
performed this task under randomized "easy" and "difficult"
conditions.

Reaction times for easy stimuli were significantly shorter than
for difficult stimuli (p <.0001); there were no significant
reaction time differences between groups. However, the number of
correct behavioral responses was significantly less for the HR
group for easy (p < .001) and difficult stimuli (p < .001).
The entire raw data set was subjected to a principal component
analysis with varimax rotation using the covariance matrix
(PCAV). Basis waveforms were extracted, and the component scores
for each of the four factors were then subjected to an analysis
of variance (ANOVA). Our results indicated that only the factor
representing the P3 component was significantly different between
the high- and low-risk groups; the P3 amplitudes were found to
be significantly smaller in the HR group as compared to the NC
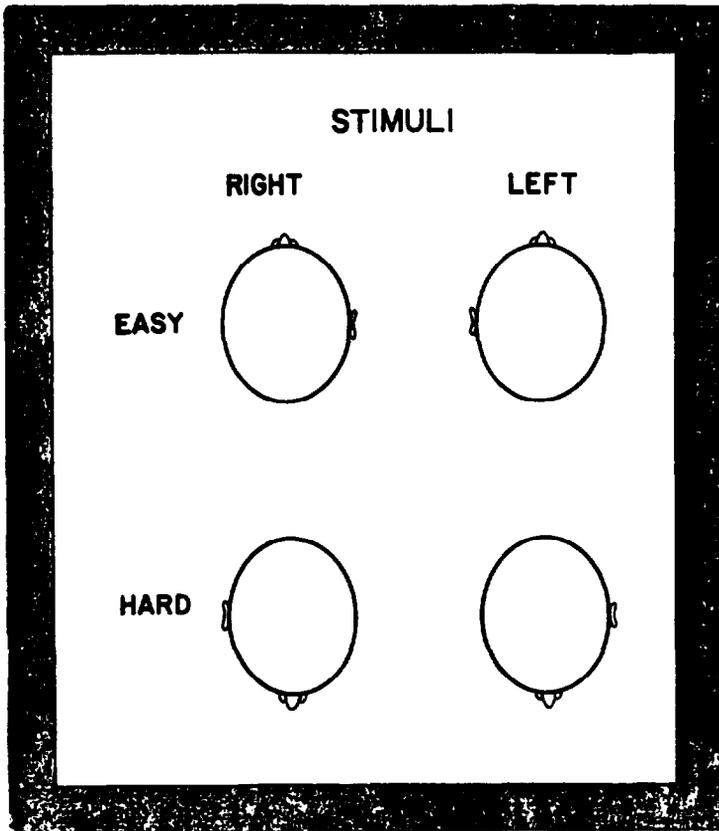group. This group difference was found to be significant at the

FIGURE 3

Experimental paradigm for head-orientation task. The target stimulus is a rarely occurring aerial view of the head with the nose and one ear drawn in, rotated in four possible positions: nose up and right ear, nose up and left ear, nose down and right ear, nose down and left ear drawn in. The nontarget stimulus is a frequently occurring oval presented in the center of a computer-generated display. In the "easy" condition, the head was facing forward (nose up on the screen) and the left ear or right ear appeared on the same side that corresponded to the appropriate button, in the "difficult" condition, the head was facing back (nose down on the screen) and the left or right ear appeared on the side opposite the corresponding button. The subject's task was to press the button corresponding to the ear present in the display as quickly as possible.

parietal electrode (where P3 is maximum) for both the easy condition (p < -01) and the difficult condition (p < .002). These findings are the first to indicate a significant difference in P3 amplitude between boys, not exposed to alcohol, who are at high risk for alcoholism and normal controls.

Differences in electrophysiological recordings in response to challenge doses of alcohol have recently been reported between males with some family history of alcoholism and control subjects. This has been reported for EEG (Pollock et al. 1983) and ERP (Elmasian et al. 1982) recordings. In the ERP study, Elmasian et al. found that male college students with family histories of alcoholism manifested different ERPs to challenge doses of both placebo and alcohol, when compared to matched controls without a family history of alcoholism; these differences between the two groups were apparent in the P3 component.

Our findings are particularly interesting as they were obtained without the use of alcohol. We found that approximately 36% of the sons of alcoholics manifested this P3 difference. However, whether these low amplitude ERPs are in fact markers for a preaisposition to alcoholism remains to be tested. Studies are under way in our laboratory to retest these children each year to determine whether those manifesting ERP differences are in fact those who actually develop problems with alcohol.

Because ot these findings, we were interested in determining whether other electrophysiological deficits observed in alcoholic patients would be apparent in boys at risk for alcoholism. Therefore, we decided to record BSPs in high-risk boys. In this study (Begleiter et al., in preparation), we examined another sample of 23 sons of alcoholic fathers between the ages of 7 and 13 with a mean age of 12.2 (S.D. = 2.1). The 23 NCs were boys who were matched for SES, age and education to the HR boys. The NC group had a mean age of 12.4 (S.D. = 2.3). The inclusion and exclusion criteria were identical to those previously described for the P3 study. Again, we only included boys without prior exposure to alcohol or other illicit drugs. As in our study with alcoholics, the latency of the tirst five positive peaks and the interpeak latencies between peak 1 and each successive peak were measured.

We did not find any differences in the auditory BSPs obtained trom sons of alcoholic fathers and those obtained from matched control subjects. The individual peak latencies and the brain stem transmission times were found to be similar in the two groups. The lack of significant differences in BSPs between HR and NC subjects is interesting in light of our observed difference in P3 between HR and NC subjects. These findings indicate that, while P3 deficits may antecede the development of alcoholism in some high-risk individuals, the brain stem deficits which we have observed in abstinent alcoholics are most probably

alcohol-related changes. Further evidence for this hypothesis is obtained by our findings that the BSP abnormalities observed in abstinent alcoholics seem to "recover" with prolonged abstinence, while the P3 deficits do not.

It remains for future research to separate those brain aberrations that antecede alcohol abuse from those that are the consequence of years of heavy drinking. It is not now known which innate differences determine responsiveness to alcohol, incluaing predisposition to alcohol abuse. Genetic differences in strains of animals have been tound to determine whether they were predisposed to drink alcohol or to find it aversive (Rogers 1972). Furthermore, differences in neurophysiological responses to alcohol have been reported in different genetic rat strains (Sorenson et al. 1980). humans have also been found to differ in their responsiveness to alcohol, e.g., augmenting/reducing (Spilker and Callaway 1969; Buchsbaum and Ludwig 1980), tamily history for alcoholism (Elmasian et al. 1982; Pollock et al. 1983). and flushers/non-flushers (Fukui et al. 1981). For example, recent findings in Japan indicate that flushers (who manifest an adverse flushing reaction to alcohol) are more susceptible to delayed BSPs than nonflushers when ingesting a challenge dose of alcohol (Fukui et al.). It is possible that the low P3 amplitudes we observe in young sons of alcoholics represent a vulnerability marker which may only become apparent in response to alcohol. For example, it is possible that, although we did not observe BSP differences between boys at high and low risk for alcoholism without the ingestion of alcohol, BSP differences may become apparent once alcohol is introduced. It may in fact be those boys who manifest P3 decrements without alcohol that will respond differently to alcohol on other evoked potential measures (e.g., BSP). Studies are under way in our laboratory to test adolescents with family histories of alcoholism under the influence of challenye doses of alcohol with a full battery of evoked potential tests.

The ability to utilize sophisticated neurophysiological tools to assess brain dysfunction in abstinent alcoholics and individuals at risk for alcoholism may prove most valuable in separating the deleterious effects of alcoholism on the CNS from the brain deficits which may antecede the development of alcoholism. The delineation of similar neurophysiological deficits in abstinent alcoholics and children at high risk for alcoholism may be of fundamental importance in the identification of possible genetic marker(s). The search for a possible cluster of neurophysiological deficits in children at high risk for alcoholism is presently under way in our laboratory.

We have recently begun to examine recovering drug addicts with the use of the same EP techniques. We reasoned that if these techniques are so sensitive in delineating the effects of alcohol on the brain (intoxication, withdrawal, long-term deficits), they would be ideal to study whether or not other substances of abuse

produce long-lasting effects on the brain.

Thus far we have examined narcotic addicts with an auditory oddball paradigm designed to examine the P3 component of the ERP. The addicts were males (mean age 35.5) who were drug-free for a minimum of 1 week. We found that recovering narcotic addicts manifest lower P3 amplitudes than matched control subjects. To the best of our knowledge, this is the first demonstration of a neurophysiological anomaly in drug addicts. These preliminary data from our laboratory run counter to the popular notion that chronic intake of narcotics may not result in CNS deficits. This popular tenet is not based on the presence of scientific evidence but has emerged in large measure in the absence ot rigorous clinical or scientific data.

The ability to assess the integrity of various CNS systems with event-related brain potentials can provide valuable clinical information about the effects of chronic drug intake on CNS activity. ERP studies of abstinent drug addicts (heroin, cocaine, PCP, amphetamine, etc.) should result in fundamental information on the neurophysiological effects of various drugs of abuse. The neurophysiological delineation of the effects of various drugs of abuse on the human brain may help elucidate the resulting pathophysiology as well as provide data on possible etiological factors.

If, as appears to be the case with alcoholism, there are antecedent brain anomalies related to drug abuse, the neurophysiological data obtained from drug abusers would be of fundamental utility in the search for possible vulnerability factors.

Our current electrophysiological data in abstinent alcoholics and subjects at risk for alcoholism, as well as our preliminary event-related potential data in drug abusers, certainly warrant further neurophysiological investigations of CNS function in drug abusers. These preliminary findings encourage us to examine recovering addicts dependent on different substances of abuse (e.g., heroin, cocaine), to investigate possible neurophysiological aberrations.

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Methamphetamine: Toxicity to Dopaminergic Neurons

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Methamphetamine (MA) is one of the prototypic psychomotor stimulants. In the past, it baa been widely used to auppreaa food intake and increase mental alertness and physical endurance. Methamphetamine and related stimulant phenethylamines can also induce an elevation in mood. This baa led to the use of the drug for nonmedicinal purposes. Amphetamine (AMPH) and related compounds have also been used to treat obesity, depression, narcolepsy, attentional deficit disorder in children, and residual symptoms of attentional deficit disorder in adults. Tolerance develops to the anorectic effects of AMPH and the dose is of ten increased; therefore, the permanent (i.e., toxic) effects of AMPH and related compounds are of concern. Among tbe undesirable effects of AMPH that occur with large doses la amphetamine-induced paycboaia. The amphetamine psychosis includes symptoms of paranoid delusions, disordered thought, inappropriate aggressive behavior, and hallucinations. When a drug history is not available, it is difficult to discriminate an amphetamine-induced psychosis from an acute psychotic episode prognostic of schizophrenia (see Costa and Garattini 1970; Seiden and Dykatra 1977; Goodman and Gilman 1980; Ellinwood and Kirby 1977).

Since the psychomotor stimulants as a class have a range of effects, their neuropharmacoloa and behavioral pharmacology, and possible neurotoxicity, have been focal points for studying interrelationahips among transmitter chemistry, drugs, and behaviors (Carlason 1970; Chiueh and Moore 1975; Creeae and Iveraen 1973, 1975). During the early 1970s, substantial evidence accumulated which suggested that the effects of amphetamines and related drugs were mediated by the dopamine (DA), norepinephrine (NE), and 5-bydroxytryptamine (5HT) transmitter systems in the brain (Moore et al. 1970; Green and Harvey 1974; Mabry and Campbell 1973; Crease and Iveraen 1975). This evidence suggested a subsequent question of whether changes in the DA, NE, or 5HT systems occur as a result of prolonged administration of AMPH

Changes in these transmitter systems were assessed in rhesus monkeys 3 to 6 months after the end of chronic MA administration. The monkeys were given high doses of MA (3.5 to 6.0 mg/kg every

3 hours, with the total daily dose between 28 and 48 mg/kg day administered intravenously. In spite of the high dose, the monkeys in these studies became tolerant to the disruptive effects of MA as measured by an operant task and feeding (Fischman and Schuster 1977). There was a loss of DA in the caudate nucleus but not in the hypothalamus, and also a small change in the level of noreplnepherine (NE) in the brain stem. The results with 5HT were not clear. The DA depletion 3 to 6 months after the last MA injection suggests that the changes in DA were permanent (tables 1 and 2).

Since the monkeys tested in the early studies were administered MA for a period of 3 to 6 months, we wondered how critical the long period of high dosing was to create the apparently irreversible damage to the DA system. Therefore, three rhesus monkeys were treated on a 2-week regimen of MA injections. These monkeys were injected with a total dose of 16 or 24 mg/kg per day divided into eight intravenous (iv) injections. The control monkeys for these studies were eight rhesus monkeys from the Wisconsin Primate Center; these monkeys had not been given any psychotropic drugs. After 2 weeks of MA injections, the monkeys were MA-free for 2 weeks and then they were sacrificed and the brains analyzed for catecholamines. As with the monkeys that were injected for longer periods of time, the monkeys injected with MA for 2 weeks shoved depletion of DA. Ye speculated that this depletion may be indicative of permanent damage to the DA system.

There is strong evidence that AMPH and related drugs bloak reuptake of DA, NE, and 5-HT; inhibit monoamine oxidase; and cause release of DA from the cytoplasmically bound pool (see Seiden and Dykstra 1977; Cooper et al. 1974). Release and blockade of reuptake would' tend to deplete levels of DA in the nerve endings in the presence of MA. However, MA-induced depletion would not be expected to last for more than several hours in the absence of MA (Ricaurte et al. 1980). Therefore, on empirical and theoretical grounds, the DA depletion seen in the monkeys weeks or months following the cessation of MA administration is not caused by the acute effects of MA.

The early researah using monkeys raised several questions: 1) Was the DA depletion Been in the rheaus monkey specific to monkeys, or would rats, mice, guinea pigs, and other animals show the same response to MA? 2) Was the depletion specific to MA, or would other psychomotor stimulants (e.g., AMPH, l ethylphenldate) that were structurally or functionally related to MA cause a similar depletion of DA? 3) Were other transmitter systems beside DA and possibly NE depleted by exposure to MA? 4) What were the minimal times and doses that would produce significant DA depletion? and 5) Were nerves destroyed, or use the synthesis and/or storage capacity of the dopaminergic cell compromised The data necessary to answer these five questions also helped to elucidate the chemical mechanism by which MA and related drugs caused the long-lasting depletion of DA and other transmitters.

TABLE 1

NE - 8 Injections of Methamphtamine (Meth.)

Daily for Several Months

Treatment	Pons-medulla	Mid.brain	Hypothalamus	Frontal cortex
Control N = 1 2	0.45 ± 0.04	0.69 ± 0.03	1.0 ± 0.26	0.21 ± 0.03
Chronic meth. 24 h poet meth. N = 5	0.23 ± 0.07(51%) $p < 0.05$	0.40 ± 0.06(67%) $p < 0.05$	0.76 ± 0.16(42%) $p < 0.05$	0.08 ± 0.23(38%) $p < 0.05$
Chronic meth. 3-6 months post meth. N = 6	0.31 ± 0.08	0.39 ± 0.07(66%) $p < 0.05$	1.4 ± 0.43	0.10 ± 0.01 (46%) $p < 0.05$

NE - 8 Injections of Methamphetamine (Meth.)

for a Period of 2 Weeks

Treatment	Pons-medulla	Mid-brain	Hypothalamus	Frontal cortex
Chronic meth. 24 h post meth. N = 3	0.21 ± 0.11(48%)	0.41 ± 0.12(69%)	1.77 ± 0.23 (98%)	0.12 ± 0.04(57%)
Chronic meth. 2 weeks post meth. N = 3	0.40 ± 0.01(88%)	0.48 ± 0.02(81%)	1.4 ± 0.34(77%)	0.11 v 0.01(62%)

TABLE 2

DA - 8 Injections of Methantphetamine (Meth.)

Daily for Several Months

Treatment	Pons-medulla	Mid-brain	Hypothalamus	Cudate	Frontal cortex
Control N = 1 2	0.16 ± 0.01	0.51 ± 0.04	0.83 ± 0.12	10.1 ± 0.57	0.09 ± 0.01
Chronic meth. 24 h post meth. N = 5	0.51 ± 0.20 ns	0.61 ± 0.11 ns	1.33 ± 0.30 ns	2.0 ± 1.0 (19%) $p < 0.001$	0.19 ± 0.04 ns
Chronic meth. 3-6 months post meth. N = 6	0.13 ± 0.03 ns	0.33 ± 0.07 ns	0.82 ± 0.17 ns	3.15 ± 0.64 (31%) $p < 0.001$	0.13 ± 0.05 ns

DA - 8 Injections of Methaphetamine (Meth.)

for a Period of 2 Weeks.

Treatment	Pons-medulla	Mid-brain	Hypothalamus	Caudale	Frontal cortex
Chronic meth. 24 h post meth. N = 3	0.19 ± 0.01	0.43 ± 0.07	1.14 ± 0.53	3.67 ± 0.50 (36%)	0.15 ± 0.02
Chronic meth 2 weeks post meth. N = 3	0.63 ± 0.90	0.76 ± 0.14	1.20 ± 0.45	2.3 ± 0.37 (23%)	0.30 ± 0.01

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Prolonged doses of MA caused depletlon of DA in the rat and the guinea pig as long as 8 weeks after the last adminiatration of MA. Furthermore, the depletions seen 2 weeks after discountinuing MA were not different from the depletions seen 8 weeks after diacontinuing MA (Wagner et al. 1980b; Ricaurte et al. 1980). These results, summarized in tables 3 and 4, indicate that MA toxicity la a generel phenomenon occurring in the rhesus monkey, mice, rata, guinea pigs, and cats (Steranke and Sanders-Bush 1980; Levine et al. 1980). Since the MA-induced toxic response (DA depletion) occurs in severel species of animals, it may also be an important side effect of chronic administration or high doses of psychomotor stimulants in humans.

The duration of the depletion suggested that nerve cells or nerve terminals might. be degenerating, but decreased storage and/or synthesis could else account for the depletions. Schmidt and Glbb (1982) and Hotchkies and Gibb (1980) found lower total activity of both tryptophan hydroxlylase and tyrosine hydroxylase in rata end mice that had received high doses of AMPH or MA then in control animals Theae two enzymes are rate-limiting for 5HT or NE and DA synthesis, and the decreased synthetic rate could account for the decrease in steady state levels However, these enzyme kinetics are also consistent with oell or terminal loss. Eillson et al. (1978) provided morphological evidence for degeneration in rat brain. After prolonged administration of AHPH through slow release pellets, evidence of malformed cells appeared in the striatum.

We round that methylphenidate, a drug functionally similar to AHPH and WA, did not cause changes in DA, NE, or 5HT systems in rat brain Therefore, not every anorectic psychomotor stimulent drug produces toxic effects to DA and 5HT nerve endings. In the past 5 years, we have shown that a number of drugs, including AMPH, cathinone, 3,4-methylenedioxyamphetamine (MDA), mazindol, phemylpropanolamine, fenfluramine, and diethylpropion, affect the DA, NE and/or 5HT transmitter systems. Some of the drugs are toxic at doses which are outside the human dose range. Mazindol oauaed smell depletions of NE at doses which were several hundred times the ED50 for the suppression of milk intake, but fenfluramine caused depletion of 5HT at a dose that vaa very close to the ED50 for the suppression of milk intake. Thus, baaed on the animal studies, one might conclude that the therapeutic ratio for mazindol was large, but that the ratio for fenfluramine was small. Similarly, MDA has toxic effects on 5HT neurons in the striatum and the hippocampus of rata at doses of 3 to 6 mg/kg per day (Ricaurte et al. 1985). Even a single administration of MDA at a doae similar to that used by humans self-administering the drug has long-term effects on hippocampal 5HT. In general, drugs having structural end functional features in common with AMPH and MA have toxic errecta upon related fiber ayatema within the CNS.

Using kinetic analysis of DA reuptake, we have found that the number of reuptake sites was reduoed by 50% by MA treatment. The affinity or the carrier system remained unchanged. In these

TABLE 3

Regional Brain Catecholamine Levels in Rats

	n	Caudate DA(µg/g)	Tlencephalon NE(µg/g)	Midbrain NE(µg/g)	Pons-medulla NE(µg/g)
Vehicle	10	8.67 ± 0.67	0.34 ± 0.02	0.60 ± 0.04	0.50 ± 0.03
25 mg/kg per day d-Meth-HCl	8	6.44 ± 0.88	0.29 ± 0.04	0.71 ± 0.05	0.45 ± 0.05
50 mg/kg per day d-meth-HCL	11	3.47 ±O.41	0.35± 0.03	0.66 ± 0.07	0.50 ± 0.03

  Rats received subcutaneous methamphetamine hydrochloride
  (d-Meth-HCl) for 30 days, for a total of 25 or 50 mg/kg/
  per day. Values reported are group means in ug/g tissue
  ± standard error of the mean. Pats were killed 2 weeks
  after the last injection. DA = dopamine; NE = norepinrphrine.

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TABLE 4

Regional Brain Catecholamine Levels in Guinea Pigs

	n	Caudate DA (µg/kg)	Telencephalon NE (µg/kg)	Midbrain NE (µg/kg)	Pons-medulla NE (µg/kg)
Vehicle	4	9.83 ± 0.27	0.33 ± 0.03	0.50 ± 0.10	0.30 ± 0.02
6-30 mg/kg per day d-Meth-HCL	6	4.62 ± 0.66	0.33 ± 0.08	0.42 ± 0.08	0.35 ± 0.05

  Guinea pigs received subcutaneous methamphetamine
  hydrochloride (d-Meth-HCl) for 30 days. Values reported
  are group means in ug/g tissue ± standard error of the
  mean. Guinea pigs were killed 2 weeks after the last
  injection. DA = dopamine; NE = norepinephrine.

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experiments, rata received MA for a period of 30 days. They were subsequently drug-free for a period of 2 to 3 weeks prior to sacrifice. The synaptosomes were in ubated with a buffered medium and varying concentrations of ^{3}H-DA (Snyder and Coyle 1969). The kinetic constants, V_{max} and K_m, were determined by fitting the data to a rectangular hyperbola and doing double reciprocal analysis on the beat fitting rectangular hyperbola. An approximate K_m value of 12×10^{-um} was Sound in both the control and the MA-treated animals. In contrast, V_{max} expressed in disintegrationa per minute was $7.9 \times 10^3 m$ for control animals and 5.4×10^3 for MA-treated animals, indicating a loss of DA uptake sites (figure 1). We examined the number of high-affinity dopaminergic binding sites using ^{3}H-spiroperidol and found no difference in affinity or binding sites (table 5, Ricaurte et al. 1980).

Repeated high doses of MA also produce long-lasting depletions of 5HT in the rat brain Within the 5HT system, levels are reduced in the amygdala, frontal cortex, and striatum. It should be noted that the regional pattern of reduced 5HT levels after administering MA is similar to that been after p-chloroamphetamine, a vell-known 5HT neurotoxin. After both MA and p-chloroamphetamine, the loss of 5HT synaptosomal reuptake sites occurs in much the same way as does the loss of dopaminergic uptake sites. Again, this evidence is consistent with the hypothesis that the nerve terminals are degenerating (Alcaurte et al. 1980).

Following chronic, high doses of MA levels of DA and 5HT are reduced for a prolonged period of time, the rate-limiting enzyme for synthesis is proportionately reduced, and the number of uptake sites is also proportionately reduced. Them observations are consistent with the hypothesis that the nerve. terminals degenerate but confirmation requires morphological evidence of neuronal degeneration.

We now have evidence that striatal move terminals degenerate in rata after a single high doae of MA Since a single dose produced toxicity, it was possible to check different time points using the silver impregnation technique to measure nerve termimal degeneration. The Fink end Heimer (1967) method depends upon the cell being stained with silver during the period of degeneration. Since the period of degeneration can be relatively short, it is necessary to do multiple time samples to find the correct period. The rata were killed 2 days after drug treatment since we had found from preliminary studies that the silver impregnation of degenerating rat nigroatriatal DA terminals is beat achieved after a 2-day survival period (figures 2 and 3). The reaults clearly show degeneration in the striatum suggesting that the DA fibers are degenerating. Since it was possible to block degeneration of 5HT fibers by pretreating with amfonelic acid, me were relatively certein that the obaened degenerating cells contained DA. We were also able to aee degeneration in the
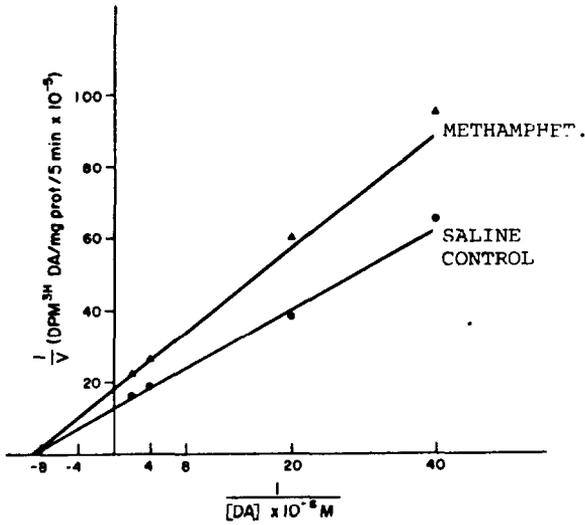
FIGURE 1

Douple-reciprocal plot of ^{3}H-dopamine uptake by
rat striatal homogenates 2-3 weeks after the high
dose methamphetamine treatment (50 mg/kg/day for
4 days). Dopamine uptake was determined at dopamine
concentrations ranging from 0.25 to 5.0 uM. The k_m
values of saline-treated (1.11 uM) and methamphetamine-
treated (0.18 uM) were not significantly different.
The difference in dopamine uptake site density (V_{max})
is significant (P<0.05). Data are from one representative
experiment replicated twice.

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TABLE 5

Specific Binding of ^{3}H-Spiroperidol to Striatal

Membranes Obtained from Rats.

Post-drug woising period(weeks)		B_{max} (fmol/mg prot.)	K_d (nM)
2	saline	92 ± 8	0.24 ± 0.02
	methamephetamine	107±11	0.19 ± 0.03
4	saline	127±9	0.19 ± 0.04
	methamphetamine	116 ± 6	0.22 ± 0.03
8	saline	107±12	0.21 ± 0.02
	methamephetamine	102±14	0.23 ± 0.04

Rats were treated with 100 mg/kg/day of methamphetamine
for 4 days. They were killed 2, 4, or 8 weeks after the
last injection. K_d and B_{max} values were derived from
Scatchard analyses of specific ^{3}H-spiroperidol binding
at concentrations ranging from 0.15 to 2.4 nM. None of the
differences in dissociation constants (K_d) or receptor
densities (B_{max}) between saline-treated and methamphetamine-
treated rats were significant. Each of the values shown is
the mean ± S.E.M. of 3 separate determinations.

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Press.
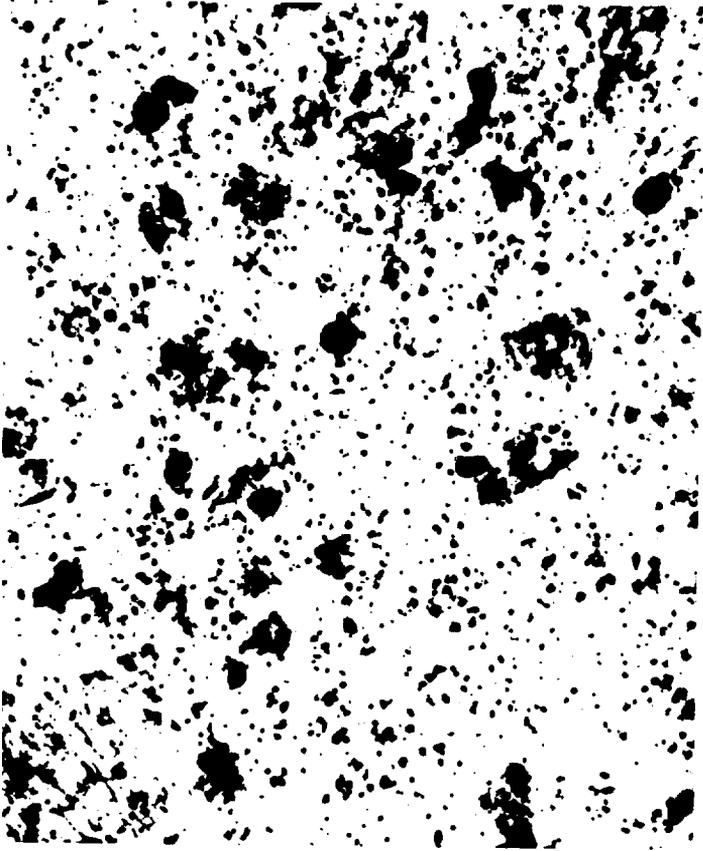
FIGURE 2

Fine granular degeneration in the rat neostriatum
following the high dose (50 mg/kg) regimen of
methylamphetamine. Four-day survival period.
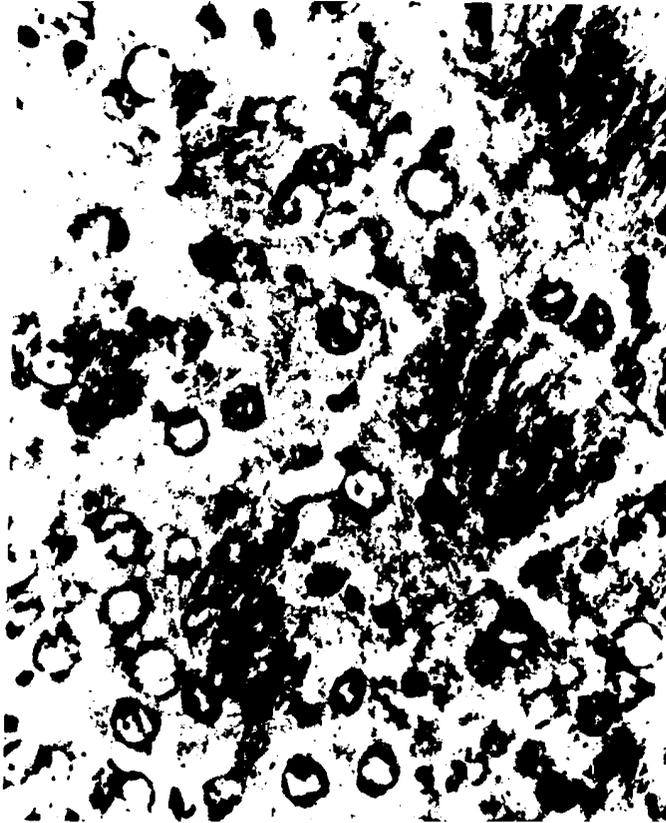Fink-Heimer method I with cresyl violet counter-
stain x 1190.

Ricaurte et al. 1982. Copyright 1982, Elsevier Biomedical
Press.

FIGURE 3

Absence of fine granular degeneration in
the neostriatum of a rat treated with saline
and killed after a 4-day survival period.
Fink-Heimer method I with cresyl violet
counter-stain x 1190.

hippocampus where there are very fev DA fibers. In addition, we found massive degenerating pyramidal cells in the sensory motor cortex. The chemical transmitter lo these cells is unknown at the present time, but it is of considerable interest that we find degeneration in this sensory motor area of tbe cortex. Chronic MA users often have sensory motor abnormalities which result lo lesions to the skin from excessive scratching or picking. Whether this is related to the degeneration of pyramidal cells is as yet unknown.

Alpha-methyltyrosine (AMT) attenuates the neurochemical changes induced by MA, but reserplne enhances these changes Thus, the AMT and reserpine results imply that the action of MA on the cytoplasmic transmitter pool may be respooslble for the MA-induced neuronal damage. AMT reduces the cytoplasmlc transmitter pool through synthesis inhibition whereas reserplne increases the cytoplasmlc pool through destruction of the transmitter storage vesicles. It has been suggested that MA preferentially releases these cytoplasmlc transmitters by reversing the direction of the high-affinity transport pump (Raiteri et al. 1979). Reserploe and AMT evidence suggests that the MA-induced toxicity is dependent upon cytoplasmic DA stores. Therefore, we reasoned that massive DA release induced by MA may in some way be neurotoxic.

Senoh and Witkop (1959) and Seoob et al. (1959) showed that DA could be non-ensymatically converted to tribydroxyphenethylamines such as 2,4,5-tribydroxyphenethylamine (6-hydroxydopamine, 6-OHDA). If DA is released by MA and MA also blocks both DA reuptake and monoamine oxldase (MAO), the amount of DA in the synaptic cleft would be large, and an oxidative reaction could occur. To test this hypothesis, male rate vere injected with 100 mg/kg of MA and than sacrificed after a time interval of either 20 minutes, 30 minutes, 1, 2, 4, 8, 16, or 24 hours. We found that between 30 minutes and 2 hours, 6-OHDA vas formed in the caudate nucleus. The 6-OHDA amounted to approximately 5% of tbe DA that was present. The declining levels of dihydroxyphenylacetic acid (DOPAC) in the caudate nucleus indicated that the MA vas a strong MAO inhibitor at this dose (table 6). In addition, we injected 6-OHDA intraventricularly which causee about the same level of depletion of DA as tbe dose of MA that we used, and found that the levels of 6-OHDA vere at least roughly comparable to tbe levels of 6-OHDA formed after MA Further experimentation showed that the AMT attenuates tbe effects of MA and we, therefore, reasoned that AMT could prevent tbe formation of 6-OHDA. This hypotbesis was confirmed (as shown in figure 4).

In summary, we bare shown that MA is toxic to DA and 5HT fibers in the central nervous system and that this toxic response is manifested by degenaration of nerve terminals. Furthermore, the toxicity to the DA system is mediated by DA itself which is oxidized to 6-OHDA due to increased release from the vesicles and blockade of inactivation. Whether this conversion of DA to 6-OHDA in vivo is a normal metabolic pathway that can occur in the

TABLE 6

Levels of 6-OHDA and DOPAC in Rats Treated with

100 mg/kg Methylamphetamine

Treatmnet	Time of Kil	mg/kg tissue			
		6-OHDA	DA	DOPAC	DOPAC/DA
Saline N = 8	20 minutes/ post injection	0 0	8.1 ±0.25	0.87 ±0.09	10.6
100 mg/kg N = 8	30 minutes/ post injection	0.20 ±0.17	8.7 0.68	0.72 0.07	10.2
100 mg/kg N = 5	1 hours/ post injection	0.39 0.31	6.1 0.60	0.62 0.07	1.3
100 mg/kg N = 6	2 hours/ post injection	0.24 0.21	6.4 0.64	0.44 0.56	6.8
100 mg/kg N = 5	4 hours/ post injection	0 0	5.7 0.65	0.37 0.08	6.4
100 mg/kg N = 6	8 hours post injection	0 0	6.9 0.54	0.33 0.04	4.8
100 mg/kg N = 4	16 hours post injection	0 0	6.3 2.6	0.78 0.25	12.4
100 mg/kg N=10	24 hour/ post injection	0 0	4.1 1.2	0.77 0.29	18.8
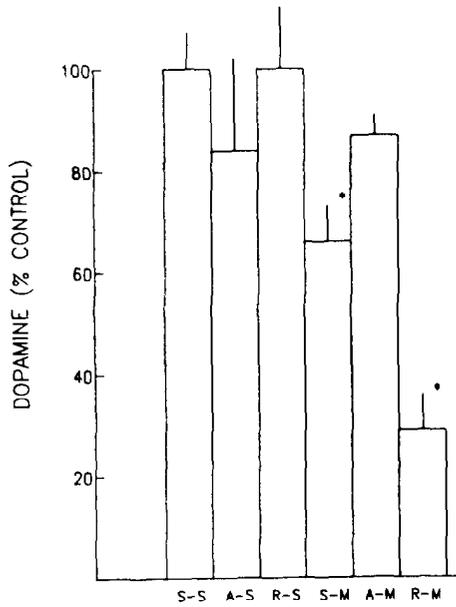
FIGURE   4

   Caudate dopamine levels for 6 groups of rats
   treated with methamphetamine (M) (100 mg/kg/day
   for 4 days) or with saline (S) (2 ml/kg for 4
   days).   Reserpine (R), aiphamethyltyrosine (A),
   or saline (S) were administered as pretreatment
   agents (see text). * = Significantly different
   from S-S group.

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absence of a drug, is certainly an interesting question and one that needs to be pursued. Several diseases, including Parkinson's disease and Huntington's chorea, are marked by the degeneration of the dopaminergic system. Whether a toxic metabolite of DA is responsible for this is a question that romaine open, but certainly in light of the data that we have promoted, it is a reasonable possibility.

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Molecular Mechanisms of Opiate Tolerance and Dependence in a Clonal Cell Line

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The neuroblastoma x glioma NGl08-15 hybrid cell line provides a model system of neuronal origin for studying molecular mechanisms of opiate tolerance and dependence. This hybrid cell line has retained many neuronal properties, including excitable membranes and light and dense core vesicles (Hamprecht 1977), thus serving as an in vitro system suitable for biochemical investigations. particular relevance to opiate research is the fact that the neuroblastoma x glioma NGl08-15 hybrid cells contain specific opiate binding sites of the delta subtype (Klee and Nirenberg 1974; Chang et al. 1978) These receptors bind enkephalin and its D-Ala^2 analogues high aff affinity (Law et al. 1983a). The effects of opiates have been well studied in this system. The opiate receptor is coupled to adenylate cyclase (Sharma et al. 1975; Traber et al. 1975). When opiate agonists are given acutely, intracellular cyclic AMP levels are lowered (Klee et al. 1975), an effect that is stereospecific and reversed by naloxone (Traber et al. 1975). With longer exposure to opiates, in contrast, the intracellular cyclic AMP level rises over a 24-hour period until it is equal to the baseline level prior to opiate exposure (Sharma et al. 1975; Klee et al. 1975; Lampert et al. 1976; Sharma et al. 1977). At this point, if the opiate is withdrawn, or naloxone is added, an immediate increase or rebound above baseline levels of cyclic AMP is observed (Sharma et al. 1975). This normalization of cyclic AMP levels after chronic opiate exposure , and the increase above baseline levels after opiate removal, have similarities to the phenomena of tolerance and dependence, respectively, that are observed when whole animals are administered opiates chronically. Thus, to some extent, these cellular phenomena may serve as useful models of the whole animal effects. Biochemical

investigations based on this view are subsequently discussed.

When it was first observed, the development of tolerance to opiate inhibition of adenylate cyclase in NG108-15 hybrid cells was considered a simple homeostatic process, involving compensatory increase in adenylate cyclase activity which brought intracellular cyclic AMP back up to baseline levels (Sharma et al. 1977). According to this line of thinking, the opiate receptors are still coupled to and inhibit adenylate cyclase, but a compensatory increase in intrinsic activity of adenylate cyclase masks this inhibition. Thus, when the opiate inhibition is removed by addition of naloxone, the compensatory adenylate cyclase activity would immediately manifest itself as an increase above baseline of intracellular Cyclic AMP levels. In this model, tolerance and dependence are intimately related and mediated by the same mechanism.

Further research, however, brought this model into question. The underlying hypothesis predicts that the degree of tolerance should directly correlate with the observed increase in adenylate cyclase activity after naloxone addition. This was not the case, however, when NGl08-15 cells, chronically treated with 10nM etorphine, were compared with cells treated with 1nM etorphine. Since a greater degree of opiate inhibition had to be reversed with the former treatment, the naloxone-induced increase adenylate cyclase activity should have been greater, but in fact it was the same as with the latter treatment (Law et al. 1982a).

Additional data also indicate that these two processes--loss of opiate inhibition and naloxone-induced increase in adenylate cyclase activity--are not closely correlated. For instance, the conditions of chronic opiate treatment necessary to elicit these processes vary. During a 24-hour treatment with etorphine, 50% of the maximal increase in adenylate cyclase activity occurred at a dose of 1 nM, which was significantly lower than the 14nM dose required for 50% of maximal tolerance (Law et al. 1983b). In a related cell line--neuroblastoma N18TG2--the two processes are completely dissociated. In these cells, chronic opiate treatment results in a loss of opiate inhibition of adenylate cyclase, but the addition of naloxone at that point does not lead to an increase in adenylate cyclase activity above baseline levels (Law et al. 1982b). All of these data, therefore, suggest that loss of opiate inhibition and the

naloxone-induced increase in adenylate cyclase activity are independent processes and their molecular mechanisms should be investigated separately.

POSSIBLE MECHANISMS OF OPIATE TOLERANCE

If loss of opiate activity is not due to a compensatory stimulation of the effector, in this case adenylate cyclase, then the most attractive explanation is that some change in the opiate receptor occurs. For example, there could be a decrease in the number of receptors, known as down-regulation. Investigations of this possibility have indeed revealed a significant decrease in opiate binding sites after chronic treatment with opiate agonists (Law et al. 1982a; Chang et al. 1982) . Further studies demonstrated that, during the down-regulation, the opiate receptors are internalized; they were associated with material co-migrating with a lysosomal fraction on a Percoll Gradient (Law et al. 1984). Studies of other receptors which internalize, such as the low-density lipoprotein (LDL) and insulin receptors, have shown that this process is part of the normally occurring recycling of the receptor, from the cell surface, into the cell, and then back out to the cell surface. The available data, interpreted in this light, suggest that chronic opiate treatment interferes with this recycling in such a manner that the number of receptors the cell surface decreases. Some data suggest that there is a decrease in the return of receptors to the cell surface (Law et al. 1985).

Down-regulation of opiate receptors does not, however, appear to fully explain how opiate tolerance is achieved. Down-regulation of the opiate receptor is not necessary for loss of opiate inhibition of adenylate cyclase. Some opiate ligands act in this system as partial agonists, such as 1 evorphanol , cyclazocine, and morphine: they induce opiate tolerance but do not, unlike full opiate agonists, down-regulate the receptors (Law et al. 1983b). Furthermore, when full opiate agonists such as etorphine are used, the loss of opiate inhibition develops before down-regulation is observed (Law et al. 1982a; Law et al. 1983b). In other words, a two-step process seems to exist. First, the receptor's affinity for opiate agonists decreases about tenfold: this occurs without a change in receptor density. Subsequently, the density of opiate receptors decreases to approximately 20 to 30% of control levels. If the

chronic opiate is then removed, the cells
resensitize in a similar stepwise fashion: first,
opiate inhibition of adenylate cyclase reappears;
and second the density of receptors increases.
Thus, while down-regulation may contribute to opiate
tolerance, it does not always accompany and usually
follows the loss of opiate activity.

As discussed above, the initial change induced by
chronic opiate treatment, and the change best
correlated with loss of opiate inhibition of
adenylate cyclase, is a decrease in receptor
affinity for opiate agonists. This loss of affinity
suggests that the receptor is being uncoupled from
adenylate cyclase, as described by Lefkowitz et al.
(1980) for beta-adrenergic receptors. In this view,
when the opiate receptor is coupled to adenylate
cyclase, it is in a conformational state with high
affinity for agonists. As the receptor is
uncoupled, it changes to a conformational state with
low affinity for agonists. Agonists are able to
induce the high-affinity-coupled state, while
antagonists bind the coupled and uncoupled receptors
with equal affinities and cannot induce one or the
other state.

In this model, tolerance blocks receptor coupling
with adenylate cyclase, keeping the receptors in a
low-affinity-uncoupled state. Binding studies
provide support for this model. when NG108-15 cells
were treated for 3 hours with 100nM D-Ala
D-Leu[5] enkephal in (DADLE) opiate tolerance
developed prior to the occurrence of significant
down-regulation of the receptors. Competition
binding to membranes of these tolerant cells was
compared to that of control cells. High- and
low-affinity sites, representing 75% and 25% of the
total binding sites, respectively, were revealed by
computer analysis of the binding data from control
membranes. In contrast, membranes from cells
chronically treated with opiates demonstrated a
shift of binding sites from high-affinity sites, now
only 45% of the total, to low-affinity sites, now
55% of the total (table 1). Presumably, in the
tolerant state receptors are shifted into an
low-affinity-uncoupled state in which the opiate
receptors cannot mediate opiate inhibition of
adenylate cyclase. These low-affinity sites, unlike
those in control membranes cannot be shifted back
into high-affinity sites by the presence of Mg^{++}
(table 1).

TABLE 1

opiate Receptor Affinities for D-Ala[2], D-leu[5]
Enkephalin (DADLE) in Control and Opiate-Treated
NG108-15 Cells

Binding in Presence of 10mM Na+ and 100um GTP	Control	100nM DADLE (24°C, 4hr)
High-Affinity Sites		
K_d (nM)	2.5+0.3	2.0+0.6
% of total sites	75	45
Low-Affinity Sites		
K_d (nM)	206.5+49.9	663+104.4
% of total sites	25	55

Binding in Presence of 10mM Mg++	Control	10nM DADLE (24°C, 4hr)
High-Affinity Sites		
K_d (nM)	1.88+0.16	0.6+0.15
% of total sites	100	49
Low-Affinity Sites		
K_d (nM)	-	280+240
% of total sites	-	51

Opiate-treated cells were exposed to 1OnM or 100nM
DADLE for 3 or 4 hours at 24°C, which was removed
by repeated washings before binding studies.
Affinities were determined by computer analysis of
competition binding studies carried out at 24°c
for 90 minutes in the presence Of 1OmM Na^{+} and
100 uM GTP or 10mM Mg++. Opiate receptors were
labeled with ^{3}H-diprenorphine, 2nM, in the
presence of various concentrations of DADLE.

An additional feature of this model is that opiate
inhibition of adenylate cyclase is now thought to be
mediated through a transducer protein, which binds
guanine nucleotides. various receptor systems,
including the beta-adrenergic, alpha$_2$-adrenergic,
prostaglandin, muscarinic and opiate, all regulate
adenylate cyclase through such nucleotide-binding
proteins, or N proteins. The regulation is negative
or positive, depending on whether the receptor
system couples with, respectively, an inhibitory
(N_i) or stimulatory (N_s) protein (Gilman 1984).
Thus, the opiate receptor, in its high-affinity

484-443 0 - 85 - 5

state, couples with the N_i protein, which mediates the inhibition of adenylate cyclase.

It follows, then, that during tolerance the opiate receptor is unable to couple the N_i protein, either because of an alteration of the receptor or of the n protein. Alteration of the receptor seems more likely since evidence indicates that the N_i protein can still mediate adenylate cyclase inhibition during opiate tolerance. For example, if NG108-15 cells are treated for 24 hours with 10nM etorphine, they become completely tolerant to opiate inhibition of adenylate cyclase, but the ability of $alpha_2$-adrenergic and muscarinic a agonists to inhibit adenylate cyclase remains uneffected (Law et al. 1983b). Since the inhibitory actions of these latter agonists are also mediated through the N. protein, opiate tolerance appears to leave the N_i protein intact.

This suggests, then, that receptor uncoupling results from an alteration of the receptor itself. In order to explore this possibility, chemical requirements for tolerance were defined in a cell-free system. Purified plasma membranes were prepared from NG108-15 cells and chronically treated with opiates in a chemically defined incubation mixture (Louie et al. 1984).

Tolerance to Opiate inhibition of adenylate cyclase was demonstrated to be dose, time, and temperature dependent. The presence of both Mg^{++} and Na^+ were absolutely reququired for tolerance development. Since these ions are necessary for coupling, the development of tolerance may require coupling of the receptor. This is consistent with the fact that antagonists, which do not induce coupling, do not cause tolerance.

Other data obtained with the cell-free system suggest that a decrease in phosphorylation, or a dephosphorylation, may be important to the development of tolerance (Louie et al. 1984). Conditions which favored dephosphorylation potentiated tolerance development. An association between opiates and a decrease in phosphorylation has been previously described using synaptic membranes from rat striatum (O'Callaghan et al. 1977; Clouet and O'Callaghan 1979). In this system, chronic morphine treatment, in vivo, resulted in as much as a 55% decrease in phosphorylation in vivo by endogenous protein kinases of particular protein bands. These changes only occurred if the morphine treatment had been chronic. Thus, the observed

decrease in phosphorylation appeared to correlate with tolerance to an opiate. Further studies will be required, of course, to investigate the possibility that dephosphorylation of the receptor has direct relevance to the uncoupling of the opiate receptor during tolerance.

POSSIBLE MECHANISMS OF OPIATE DEPENDENCE

As discussed above, the naloxone-induced increase in adenylate cyclase activity does not correlate well with the development of tolerance and may be viewed as a different adaptive process occurring during chronic opiate treatment. A mechanism is required to account for how the displacement of an opiate, from its binding site on an opiate receptor, results in the stimulation of adenylate cyclase. Such a mechanism presumes that the receptor remains coupled to some effector which can directly or indirectly stimulate adenylate cyclase. If during tolerance development the opiate receptor becomes uncoupled from the N_i protein, then N_i must not be directly involved in mediating the naloxone-induced increase in adenylate cyclase. Some other mechanism involving other cellular components should be sought.

One hypothesis is that some cytosolic component which accumulates during chronic opiate treatment stimulates adenylate cyclase. This was suggested by experiments in which membranes and cytosol from control and opiate - treated cells were isolated and then mixed in every possible combination. The cytosol from opiate-treated cells provided greater stimulation of adenylate cyclase in membranes than did cytosol from control cells (Griffin 1983). Analysis of cytosol from chronic opiate-treated cells indicated two components capable of stimulating adenylate cyclase, both individually, and synergistically when combined (table 2).

One of the cytosolic components was determined to be adenosine. Its stimulation of adenylate cyclase was inhibited by treatment with adenosine deaminase or with the adenosine antagonist, theophylline. Furthermore, neither this cytosolic component nor exogenous adenosine stimulated adenylate cyclase in NlBTG2, a neuroblastoma cell line which does not demonstrate a naloxone-induced increase in adenylate cyclase activity.

TABLE 2

Characterization of Cytosolic Components from
NGl08-1 Cells Chronically Treated with Etorphine

	Component 1	Component 2
Molecular weight	300	>10,000
Heat stable	+	+
Calcium dependent	−	+
Stimulation of adenylate cyclase as % of control	220	280
Putative identity	Adenosine	Calmodulin

Cells were exposed to 1uM etorphine for 4 hours.
Then cytosol and membranes were separated and the
former was resolved into two components, which
stimulated adenylate cyclase, by gel chromatography
with a Bio-Gel P-6DG desalting column. Stimulation
of adenylate cyclase in the presence or absence
(control) of the components was determined using
chronically etorphine-treated membranes in the
presence of 400uM naloxone. When components 1 and 2
were combined, the stimulation of adenylate cyclase
was 670% of control.

The exact relationship between increased
intracellular adenosine levels and naloxone-induced
increases in adenylate cyclase activity remains
unclear. If it is assumed that intracellular
adenosine increases because opiates prevent its
release from NG108-15 cells, however, one could
postulate that during chronic opiate treatment,
intracellular adenosine gradually accumulates. Upon
addition of naloxone and, thus, antagonism of
opiate inhibition of adenosine release, the
adenosine would be released into the extracellular
space. Then, these high levels of adenosine would
stimulate the cell's adenylate cyclase through the
adenosine receptors (A_2) on the cell surface.
This naloxone-induced induced increase in adenylate cyclase
activity would not be observed after acute opiate
treatment because the accumulation of intracellular
adenosine would be insignificant at that point.

In support of the above hypothesis, some studies
indicate that the basal adenylate cyclase activity
is in part maintained by continuous release of

adenosine from NG108-15 cells (Griffin 1983). If chronic opiate treatment disrupted this release of adenosine, the development of adenosine receptor super-sensitivity might be expected. This, in fact, was demonstrated by the finding that chronic opiate treatment is accompanied by an increase in the coupling of adenosine receptors (Griffin 1983). This increase in adenosine-receptor coupling and the increased levels of adenosine might synergistically stimulate adenylate cyclase after naloxone addition, As predicted by the hypothesis, the prior desensitization of the cells to the effects of adenosine, by chronic adenosine treatment, abolished the naloxone-induced increase in adenylate cyclase activity (Griffin 19831.

The second cytosolic component from opiate-treated cells was found to be heat stable and calcium dependent, and to have a molecular weight of greater than 10,000 daltons. While this component has not been rigorously identified, it resembles calmodulin in many of its properties. In particular, this cytosolic component's stimulation of adenylate cyclase is enhanced greatly by the presence of calcium. Similarly, naloxone-induced increases in adenylate cyclase activity can be Effected by manipulation of the intracellular level (Griffin 1983). If this component is calmodulin, these findings would be consistent with the reports that calmodulin may act directly on adenylate cyclase's catalytic subunit (Salter et al. 1981). Opiate treatment has been shown to decrease the amount of calmodulin bound to the plasma membrane of NG108-15 cells and this might influence calmodulin stimulation of adenylate cyclase (Nehmed et al. 1982).

Any proposed mechanism of dependence must involve an opiate receptor that is coupled to an effector system which will directly or indirectly stimulate adenylate cyclase when naloxone is added. This system might involve adenosine, calmodulin, or other unidentified components. This implies that the opiate receptor remains coupled to effectors responsible for dependence while, simultaneously, being uncoupled to effectors involved in tolerance development. This hypothesis is not unreasonable since receptors may interact with multiple effectors and these interactions may be effected differentially by chronic drug treatment. Studies have indicated that while the opiate receptor may be completely uncoupled from the inhibitory-nucleotide binding protein (N_i), it is still coupled to other N proteins, whose functions are as

yet unidentified. When NG108-15 cells are pretreated with pertussis toxin, the N_i protein is uncoupled from opiate receptors, yet opiate binding is still sensitive to the addition of guanine nucleotides, like guanosine triphosphate (GTP) (Law & Loh, unpublished). This suggests that the receptor is still coupled to other guanine-nucleotie-binding proteins, because the decrease in opiate binding, caused by guanine nucleotides, is generally mediated through an N protein. Similar binding data are obtained from cells chronically treated with DADLE. In this case the opiate receptor is uncoupled from the N. protein during tolerance development, but appears still to be coupled to other N proteins. These other N proteins may be involved in mediating the dependence aspects of chronic opiate effects.

CONCLUSION

The molecular mechanisms of tolerance and dependence appear to be extremely complex. A unitary mechanism to explain both processes has not been found and some investigations have indicated that separate mechanisms are in fact involved. The neuroblastoma x glioma NG108-15 hybrid cells provide a relatively simple model system with which these complex mechanisms may be elucidated. Only until some understanding is mastered on this single cell level, will more intricate brain systems be comprehensible.

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Consequences of Perinatal Drug Administration on Brain Development: Striatal Opiate Receptors and Dopamine

Sandra Lynn Moon

INTRODUCTION

Perinatal exposure to certain drugs and other agents can alter
the development of the organism, and the consequences of these
alterations may be long lasting. We have become more sophisti-
cated in the analysis of these effects because we are beginning
to look at the subtle molecular changes in multiple systems in
addition to gross mutagenic capacity (from Nair 1974). The
following study outlines the normal development of mu opiate
receptors and dopamine in the striatum and demonstrates that
after prenatal administration haloperidol induces changes in
the normal course of development.

The striatum is an ideal model for examining drug-induced
changes. The corpus striatum is now recognized as a hetero-
geneous structure that can be compartmentalized on the basis of
its diverse neurochemistry, cytoarchitecture in some animals,
and anatomical connections (see Moon Edley and Herkenham 1984a).
In experimental animals, these compartments are: (1) var-
iably shaped zones called "patches", including a band along the
dorsolateral perimeter of the structure, and (2) the zone
outside the patches, called the "matrix." In the rat, the
distribution of mu opiate receptors is an excellent marker for
the striatal compartments because these receptors appear very
early in striatal development, and are quickly transformed
into dense patches on a sparsely labeled matrix. This pattern
is maintained from a late prenatal age throughout adulthood.
Although dopamine is evenly distributed in the adult rat
caudate-putamen, during early development dopamine is clustered
in so-called "islands" (Loizou 1972; Olson et al. 1972). All
striatal topographic discontinuities are not in alignment,
however (e.g., see Graybiel et al. 1981), and the topographic
relation of the transient dopamine "islands" with opiate
receptor patches had not been established, even though chemical
systems that share a compartment might be expected to have
functional links.

THE NORMAL DEVELOPMENT OF STRIATAL OPIATE RECEPTORS AND DOPAMINE IN THE RAT

To investigate further the development of the compartmentalization of opiate receptors and dopamine in the striatum, the temporal and spatial sequences of these two markers were examined in the same brain. In the rat, the first striatal cells are born on the 12th day in a gestational period that lasts 22 days. By means of glyoxylic acid-induced catecholamine fluorescence (De la Torre 1980) and in vitro receptor autoradiography using emulsion-coated tissue sections and tritium sensitive film (Herkenham and Pert 1982), dopamine and opiate receptors labeled with either ^{3}H-naloxone or ^{125}I-enkephalin were compared on alternate striatal sections beginning at embryonic day (E) 13 through adulthood.

The Caudate-Putamen

Opiate receptors and dopamine, which are two of the earliest appearing striatal neurochemical markers, emerge in the caudate-putamen 1 full week before birth. At that time, neither is arranged in patches. The nigral fiber system, the source of striatal dopamine, appears to be the earliest striatal afferent connection (see Moon Edley and Herkenham 1984a). Other components related to these striatal chemical systems arrive later. For example, ^{3}H-DALA enkephalin-labeled opiate receptors (Kent et al. 1982) arrive on E18, and enkephalinlike immunoreactivity is not apparent until E20 (Pickel et al. 1981). Striatal dopamine receptors labeled by ^{3}H-spiperone do not arrive until E18.

On E14, dopamine fluorescence is dim and scattered throughout the caudate-putamen. Small irregularly shaped clumps of fluorescence have no resemblance in size or position to the islands that have yet to emerge. Opiate receptors appear on E14, also. They are distributed throughout the caudate-putamen with a slightly greater density in the caudal portion. Some opiate labeling is seen in the subependymal layer among cells that have yet to migrate into the striatal anlage.

The first signs of a discontinuous pattern appear a few days before birth. On E19, dopamine begins to aggregate along the lateral striatal perimeter (figure 1A) and patches are apparent on E20 (figure 1B). High density opiate receptor patches and a band along the perimeter emerge on E20 (figure 2). At the earliest time of striatal heterogeneity, opiate receptor and dopamine patches appear to be aligned, although the vague boundaries of the aggregates make an accurate determinate of the degree of alignment difficult. From this point on, opiate receptor and dopamine developments are nearly synchronous. Dopamine patches are at the height of their visibility on postnatal day (P) O. Figure 3 shows that they stand out as aggregates composed of bright fluorescent dots on a dark and dimly fluorescent matrix.

Dopamine patterning is generally more distinct in the lateral aspect of the striatum. By contrast, opiate receptor patches, although quite distinct on P0, are still embedded in a well-labeled matrix. There is now a clear one-to-one correspondence between dopamine patches and opiate receptor patches in adjacent tissue sections, as seen in figure 4.

During the second postnatal week, dopamine fluorescence in the caudate-putamen loses much of its dotted quality, moderates in intensity in the patches, and increases in intensity outside the patches. These events, which begin to obscure dopamine patches, will lead to their eventual disappearance. On the other hand, the opiate receptor pattern continues to mature into discrete and dense patches with definite borders on a lightly labeled matrix. By P16, opiate receptors in the patches and matrix closely approximate the adult distribution, while nearly all dopamine fluorescent patches have become obscured. At this late stage in the life of dopamine patches, an occasional patch can be discerned, and it is still aligned with an opiate receptor patch (figures 5A and 5B). But, by P18, the dopamine fluorescence in the caudate-putamen is virtually homogeneous, except for a bright stripe along its medial border (figure 5C).

It has been suggested that dopamine plays a role in striatal development (Pickel et al. 1981). Because the existence of dopamine patches spans most of the period of striatal development, a role as a developmental cue is suggested, particularly for those striatal systems that acquire the same patch/matrix scheme. However, since dopamine and opiate receptor developments are nearly coincident, one can only speculate about a developmental role for either opiate receptors or dopamine in the topographic arrangement of striatal compartments.

The Nucleus Accumbens

Whereas the caudate-putamen has strong sensorimotor ties, the nucleus accumbens is thought to be limbic-related. Their connections are generally in parallel, although distinguishable. Their neurochemistry is extremely similar.

The development of the nucleus accumbens lags behind that of the dorsal striatum. It has some features in common with the caudate-putamen, but there are some major differences as well. Both dopamine and opiate receptors are evenly distributed when they first arrive: opiate receptors on E18 and dopamine on E20. Just as in the caudate-putamen, opiate receptors coalesce into dense patches that are apparent by P6 (figure 6B). The dopamine fluorescence pattern, however, is comprised of low intensity "holes" surrounded by bright and dotted fluorescence in the matrix. Thus, the major difference between the dorsal striatum and the nucleus accumbens is that in the caudate-putamen, bright fluorescence and opiate receptor-rich patches are aligned until dopamine patches are obscured; but, from the earliest stages of

patterning in the nucleus accumbens, opiate receptor-rich patches fill holes in the dopamine fluorescent distribution (figure 7). These dopamine-poor zones and opiate receptor dense patches remain aligned throughout maturity (also see Herkenham et al. 1984).

Summary

Thus, dopamine systems in the caudate-putamen and in the nucleus accumbens can be distinguished from each other by their: (1) time of appearance, (2) early pattern, (3) subsequent adult distribution, and (4) fluorescent quality at maturity. In contrast, opiate receptors obey the same rules of pattern formation in both the dorsal striatum and in the later developing nucleus accumbens.

Striatal dopamine can be subdivided on the basis of many other criteria, including source of origin, turnover rate, and susceptibility to pharmacological manipulation (see Moon Edley and Herkenham 1984a). The positive register of opiate receptors and dopamine in the caudate-putamen and the negative correlation in the nucleus accumbens is another criterion by which to subdivide striatal dopamine, and it is a contrast between the caudate-putamen and nucleus accumbens. It seems clear that the opiate and dopamine systems can be suspected of interacting differently in the compartments of the caudate-putamen, where there has been established a positive correlation between dense, opiate labeling and dopamine, and in the compartments of the nucleus accumbens where the correlation is negative. Such differences in chemoarchitecture must certainly underlie regional dissimilarities in function.

PRENATAL DRUG ADMINISTRATION

The previous study outlined the developmental period of striatal opiate and dopamine topographic organization. The role that enkephalins play in the striatum is not understood; however, numerous studies document striatal opiate/dopamine interactions. The register of opiate receptors and dopamine early in development could result from the localization of opiate receptors on dopamine terminals, as some biochemical studies have implied (Biggio et al. 1978; Pollard et al. 1977). Destruction of nigral terminals results in a large decrease in opiate receptors found in the patch zones (Bowen et al. 1982). Two alternatives could explain the loss: some opiate receptors could have a terminal localization and are thereby lost when the terminal is destroyed, or the decreased opiate binding may be mediated transsynaptically through the loss of dopamine interactions with its receptor. It is not possible to deplete rat fetuses of dopamine through the use of 6-hydroxydopamine (Imamoto et al. 1980; Lidou et al. 1982) to study the effect of the absence of dopamine on striatal development. However, interactions in the developing striatum can be investigated further by means of other pharmacologic manipulations during the time of early striatal pattern formation. The dopamine antagonist haloperidol was administered prenatally to investigate whether dopamine

receptor-mediated events are important for the normal develop-
ment and maintenance of dopamine and opiate receptor distribu-
tions (see Moon 1984).

Haloperidol-filled osmotic minipumps were implanted subcutane-
ously in pregnant Sprague-Dawley rats that had been lightly
anesthetized with ether. The pumps released haloperidol (0.71
mg/day) from E15 through P0. As controls, some pregnant rats
were implanted with saline pumps and others were left untreated.
The litters from the haloperidol-treated group were small, con-
taining usually from 3 to 7 pups, as compared to the normal lit-
ters of 6 to 12 pups. On P0 and P16, rat pups were taken from
each litter and their brains processed for _in vitro_ receptor
autoradiography and dopamine fluorescence. Sections prepared
for autoradiography were incubated in ^{3}H-naloxone, ^{3}H-spiperone,
or ^{3}H-muscimol to label opiate! dopamine, and gamma aminobutyric
acid (GABA) receptors, respectively. After film exposure, the
binding densities in sections from the anterior half of the
striatum were measured by computer-assisted optical densitometry
(Goochee et al. 1980). Optical density (O.D.) measurements were
used as estimates of binding values.[1] For the statistical anal-
ysis of drug effects, nonspecific binding--estimated as the
binding to white matter dorsolateral to the striatum--was sub-
tracted. The measurements were pooled to yield average specific
binding for each drug condition per film. Student's t-test was
used to indicate significance. In all binding conditions, tis-
sue from the group of animals pretreated with saline was iden-
tical to untreated tissue.

Dopamine Receptor Binding

That chronic antagonist treatment results in increased receptor
binding is a corollary of receptor theory that has been well
demonstrated many times in many systems, but most often in the
adult. There have been demonstrations of exceptions to this
rule. In the case of chronic dopamine antagonism in the adult
rat, striatal spiperone binding increases (e.g., Burt et al.
1976). The relationship between increased dopamine receptor
binding, the pathogenesis of schizophrenia, and abnormal invol-
untary movements is not well understood. It would be interest-
ing to know whether in the present experimental paradigm the
developing animal responded with increased binding, since
changes in dopamine receptor binding may be related to either
psychiatric or neurologic disease states.

Spiperone binding is normally arranged in a high-lateral to low-
medial density gradient in the striatum and is apparent at P0
and P16 (figure 7). Chronic haloperidol treatment did not alter
the pattern of ^{3}H-spiperone binding. However, binding was uni-
formly elevated over saline control measures at P0 by 27%
(p<0.02) and at P16 by 34% (p<0.05). These results suggest that
the neonate and the adult respond to dopamine receptor blockade
in the same way.

However, Rosengarten and Friedhoff (1979) reported that spiperone binding decreases after chronic prenatal haloperidol treatment. There are a number of differences in the experimental protocols between that study and the present one that may explain the differences in results. In particular, the region of striatum analyzed and the binding conditions are different. In the present study, only the anterior caudate-putamen was sampled and ketanserin was used to block spiperone binding to serotonin receptors, whereas it seems that the entire striatum, including nucleus accumbens, was analyzed in the previous study and no serotonin blocker was used. It is unlikely that the lack of drug wash-out contributed significantly to the variant results since, as figure 7 illustrates, 16 days after drug withdrawal (on P16). striatal spiperone is elevated by virtually the same amount (34%) as immediately following drug withdrawal on P0 (27%).

Nigral terminals were presumed intact in all cases since all brains showed normal dopamine fluorescence in the striatum. Since it has been postulated that some striatal opiate receptors are located on dopamine-containing terminals, the normal fluorescence suggests that the decrease in opiate binding was not a mere secondary effect caused by the degeneration of dopamine-containing terminals.

Opiate Receptor Binding

Haloperidol has no affinity for mu opiate receptors. Thus, changes in opiate receptor binding after haloperidol treatment presumably are due to transsynaptic interactions between systems containing dopamine and opiate receptors.

In normal animals, opiate receptor binding is arranged in patches at P0; however, the matrix binding is only slightly sparser (Kent et al. 1982). At this age, average O.D. values were taken from patch and matrix, together, to perform the analysis. In the haloperidol-treated animals, the pattern of opiate receptor binding was unaffected, but the binding density was substantially reduced. Naloxone binding in the caudate-putamen was decreased by 45% (p<0.02) on P0.

Opiate receptors have nearly reached their adult distribution by P16. At this age, separate measurements of patch and matrix binding were analyzed. At P16, in the haloperidol-treated animals, the drug-induced depression of opiate binding still lingered, but its magnitude was diminished. Moreover, patch binding was more stunted (-17%, p<0.05) than matrix binding (-5%, p<0.05), suggesting different recovery mechanisms in each of these two compartments. Likewise, opiate receptor binding is preferentially reduced in striatal patches after destruction of the nigrostriatal pathway in the adult (Bowen et al. 1982).

In the present experiment, dopamine terminals have been left intact, suggesting that the decrease in patch opiate binding is not merely due to mechanical destruction of the neuronal element

on which opiate receptors might be found, e.g., the dopamine
terminal (see discussion, Moon Edley and Herkenham 1984a). As
evidenced by the different rates of recovery of opiate receptor
binding in patch and matrix, a different recovery mechanism may
operate in each of these two striatal compartments. One sug-
gestion is that these different rates of recovery in the com-
partments may be related to different connectives. The pro-
jection to the striatum from the substantia nigra, proper, has a
patchy distribution (Wright and Arbuthnott 1981). Preliminary
evidence suggests that these patches are aligned with opiate
receptor-dense patches, and that the outlying dopaminergic cell
groups, the ventral tegmental area of Tsai, and the retrorubral
nucleus project preferentially to the matrix of the caudate-
putamen and nucleus accumbens (Moon Edley and Herkenham 1984b).

GABA Receptor Binding

It could be questioned whether these haloperidol-induced changes
represent a disruption of ontogeny due to a general vulnerability
of the developing striatum, rather than specific disruptions ow-
ing to opiate/dopamine interactions. As a test of this hypoth-
esis, some sections were used for _in vitro_ ^{3}H-muscimol binding.
Muscimol binding is also distributed in patches on P0. There
was no significant difference between haloperidol and control
tissue, although a tendency for reduced muscimol binding in
haloperidol-treated tissue was noted. Like dopamine, ^{3}H-mus-
cimol-labeled GABA receptors take on a uniform distribution in
the striatum by P16. The specificity of the haloperidol-induced
changes is therefore supported by the lack of an effect on
striatal GABA receptors.

Functional Implications

Treatment with haloperidol induces a large increase in striatal
enkephalin content in the adult (Hong et al. 1978). If the same
mechanism operates in the neonate, perhaps the reduced opiate
receptor binding shown here is correlated with elevated enkeph-
alin. Both enkephalin content (Hong et al.) and receptor binding
follow a similar course of normalization after drug withdrawal.
In this regard, it is clear that investigations of relative
transmitter levels and receptor density will be necessary to
access drug-induced changes in "set point" of individual chemical
systems and the functional implications of their regulation.

Haloperidol-induced behavioral responses are known to follow
different time courses. In man, haloperidol (1) can produce a
parkinsonian-like syndrome after treatment; (2) has antipsychotic
effects after repeated doses; and (3) can lead to the develop-
ment of tardive dyskinesia after long-term administration. With
multiple effects that follow different time courses, it is likely
that nondopaminergic systems are involved in producing some of the
haloperidol-induced actions. Some of the nondopaminergic respon-
ses may be found within the striatum. The changes in the opiate
system in the dorsal striatum suggest that striatal opiates may

have a role in these effects. In experimental animals, haloper-
idol produces catalepsy, and opiates administered into the lat-
eral ventricle also have been shown to produce a cataleptic-like
state (Bloom et al. 1976). Haloperidol is now the primary anti-
psychotic in use, and pregnant women have taken haloperidol
either as an antipsychotic or antiemetic. The present results
demonstrate that prenatal haloperidol treatment alters striatal
development. There may be functional consequences to these al-
terations, particularly since all affected systems do not recover
at the same rate and these consequences have yet to be studied.

Moreover, the present study appears to be a good model to study
the effects of changes on the development of the nervous system.
It is noteworthy that only by means of visualization techniques
have the functional units of the striatum become appreciated.
The utility of in vitro autoradiography for this type of study
is that multiple systems can be examined quantitatively with a
sensitivity comparable to standard biochemical procedures using
tissue homogenates, but at the level of the functional unit.

FOOTNOTE

[1]The extent of drug-induced changes, estimated from O.D. values,
has probably been underestimated in the study, owing to the rel-
atively low overall density values of these data (see bar in fig-
ures 7 and 8) and the properties of LKB film (see Herkenham, this
volume).

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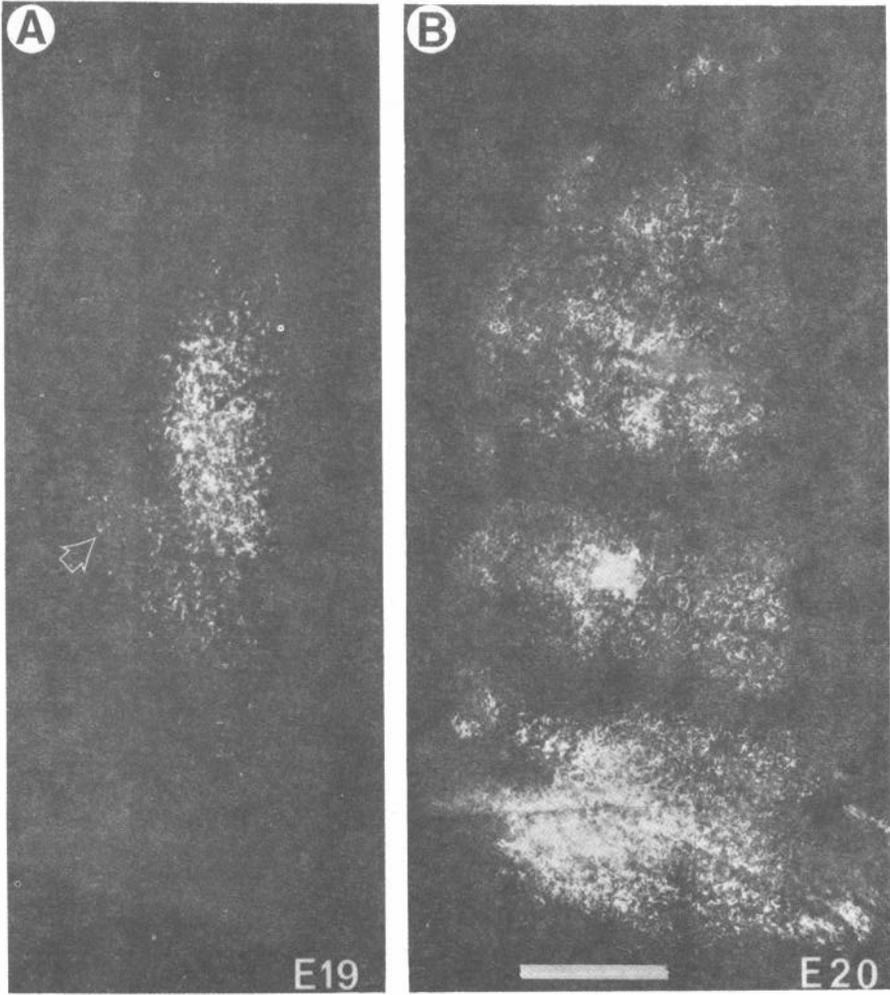
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FIGURE 1

Photomontage of the earliest signs of dopamine aggregations in coronal sections. A: arrow points to the formation of a lateral rim on E19. B: On E20, dense patches of fluorescence are apparent within the nucleus.

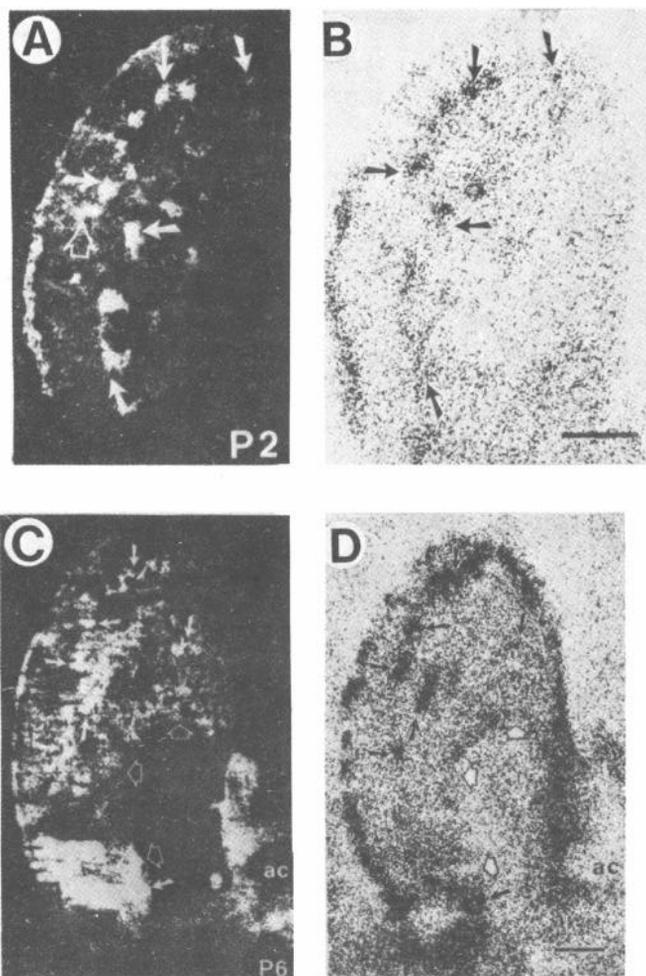**FIGURE 2**
*Emerging opiate receptor patches labeled with ^{3}H-naloxone coronal
section through the striatum on E20. Bar=500μm.*

FIGURE 3

Photomontage of dopamine fluorescent patches and a band along the lateral border in a coronal section on P0 (open arrow points to a dense aggregate of fluorescence in the caudal nucleus accumbens).

141

FIGURE 4

Patch alignment in adjacent sections on P2 (A, B) and P6 (C, D). Photomontages show dopamine fluorescent patches, at white arrows in A, C. They perfectly overlap opiate receptors highlighted by black arrows in B, D. Film autoradiographs of ^{3}H-naloxone binding in B and ^{125}I-enkephalin binding in D.
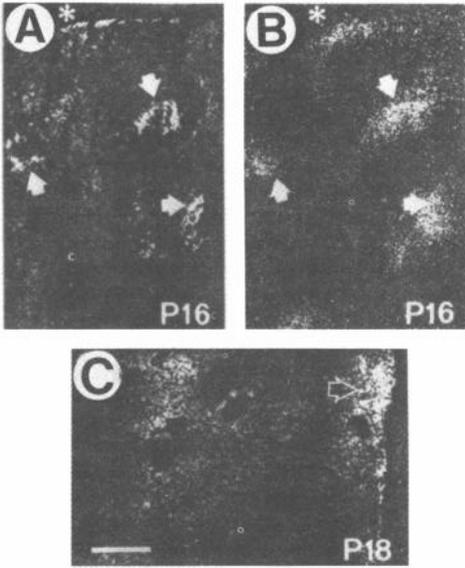
FIGURE 5

*The disapperance of domamine patches in postnatal week 3 A: arrows point to last remaining dopamine patches in dorsal striatum. B: a dark-field photomicogrpah of an adjacent section labeled with ^{125}I-enkephalin and emulsion-coated. White arrows point to opiate receptor patches that are in register with dopamine in A. Asterisks point to band along the dorsolateral striatal border. C: At P18 no dopamine patches are ap- parent. Open arrow points to bright fluorescence along medial striatal wall. Bar=200μm.*
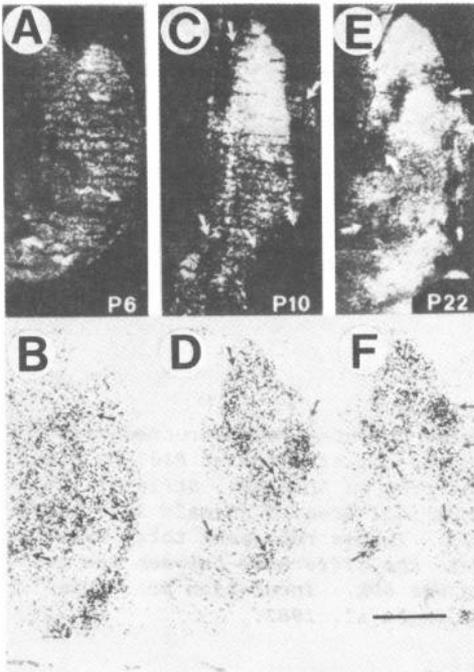
FIGURE 6

In the nucleus accumbens, dopamine at P6 (A), P10 (C), and P22 (E) shows zones of dim fluorescence (white arrows) that are in register with ^{125}I-enkephalin-labeled patches shown in film auto- radiographs from adjacent sections, B, D, F respec- tively. Bar=500μm.

143
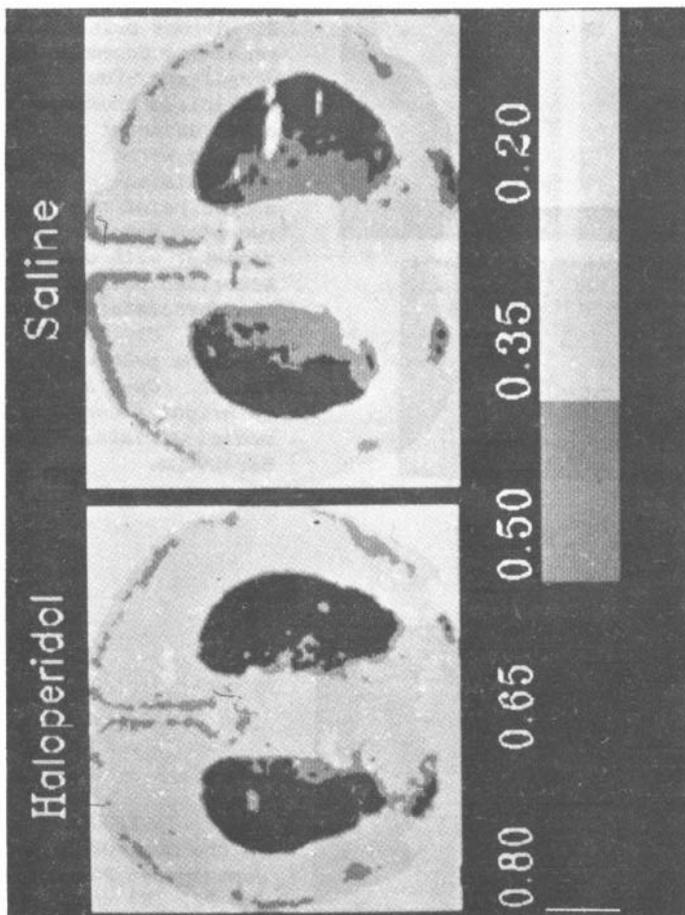
FIGURE 7

Photograph of TV monitor showing computer reconstructed film images of ^{3}H-spiperone binding to the striatum at P16. O.D.s are represented as 5 shades of gray on the bar. Striatal spiperone binding in the haloperidol-treated animals is greater than in saline-treated animals. Images represent total binding and thus visually underestimate the difference between the two conditions. Specific binding was 60%. Incubation procedure followed the protocol of Quirion et al. 1982.

144
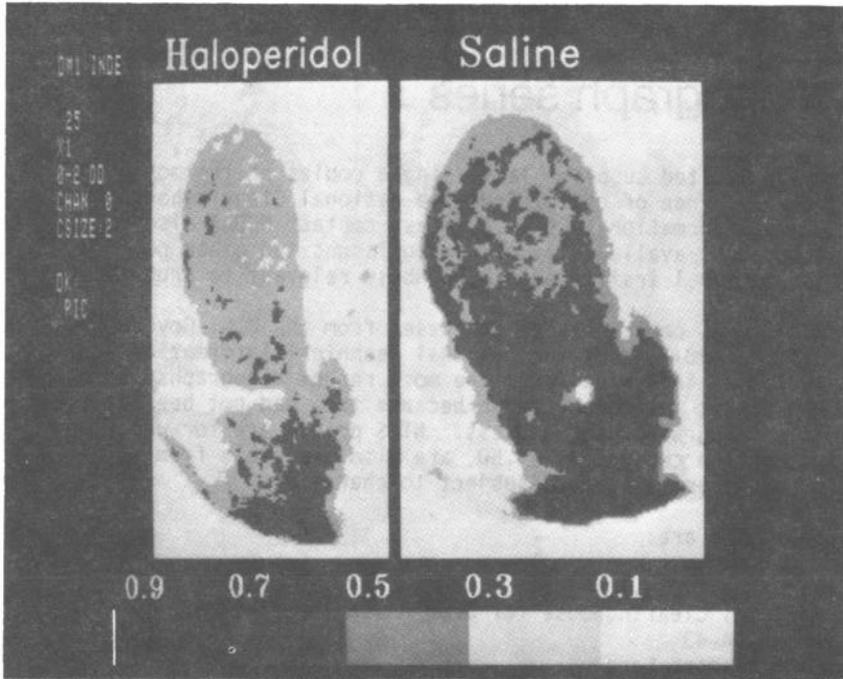
FIGURE 8

Photograph of TV monitor showing computer reconstructed images of ^{3}H-naloxone binding to the left striatum at PO. The relative O.D.s are represented in the 5-shade gray scale on the bar. When compared to saline treatment, haloperidol reduced opiate binding. Specific binding was 93%. Incubation procedures are detailed by Moon (1984). Images are of total binding. Arrows point to emerging patches.

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