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The Role of Neuroplasticity in the Response to Drugs

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The Role of Neuroplasticity in the Response to Drugs

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Neurosciences Research Branch
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The Role of Neuroplasticity in the Response to Drugs

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Preface

On September 22 and 23, 1986, the National Institute on Drug Abuse held a technical review meeting entitled "The Role of Neuroplasticity in the Response to Drugs" to review those factors that may alter the response of the central nervous system to a drug. Historically, researchers investigating the development of an increased or decreased response to a drug of abuse after its initial dose or after chronic administration have related these response changes to the continued presence of the drug. The mechanisms by which these adaptations were produced have remained largely unknown. In the last few years, however, there have been significant advances in understanding the plastic capacities of neural tissue, both in the developing nervous system and in the adult brain, that may shed light on the ability of the nervous system to alter its response to drugs. Adaptation in both the central and peripheral nervous systems may occur in milliseconds or take months, will differ from one discrete brain area to another, and will be specific for the external or internal stimulus evoking the response. Because one purpose of this technical review was to encourage the use of new ideas and methods from neuroscience in studies of abused drugs, participants included speakers who are interested in neural plasticity per se, those who are applying these concepts to response changes induced by neurotransmitters and modulators, as well as those studying drug abuse problems directly. In the hope that this commingling would encourage neuroscientists to enter the drug abuse field, and to stimulate the use of new methodologies by researchers already in the field, material generated by this meeting has already been incorporated into a program announcement by the Neurosciences Branch, Division of Preclinical Research.

The first group of speakers discussed plasticity in the developing nervous system. Because of the constant change taking place in the nervous system during development, and because the timing of developmental events depends on events that have already occurred, it is during development that the nervous system is most vulnerable to drugs. Some of the reasons for this vulnerability and

some of the consequences resulting from manipulation of the developing organism during this vulnerable period were described.

B. MCEWEN ("External Factors Influencing Brain Development") discussed the relative contributions of genetic and environmental factors in the development of the nervous system. Because environmental stimuli can act on the genome, however, the distinction between the two types of influences may, in some cases, not be particularly important. Steroid hormones, which are released by a variety of environmental stressors, act on the genome through specific brain steroid receptors linked to a second messenger that causes repression or derepression of gene activity. Through this mechanism, extrinsic or intrinsic hormones can affect the development, cyclic activation, adaptation, amount of damage, and degree of reinnervation of the nervous system. Thus, epigenetic factors found in the environment can alter the phenotypic expression of a variety of neuronal traits.

Drugs that alter the contribution of the genome to the adaptability of the nervous system will have long-term effects, whereas drugs that do not alter the normal contribution of the genetic complement will have actions of shorter duration.

J. LICHTMAN ("Rearrangement of Synapses During Development") continued the discussion of the vulnerability of the developing brain to external factors, such as drugs of abuse, by reviewing the way in which synaptic connections are made. Since synapses in different regions of the nervous system follow different rules of development, he described typical examples. At the neuromuscular junction, where, in the adult, a single nerve synapses on each muscle fiber, the maturing muscles have several axon terminals competing to occupy the end plate. As development progresses, all but one nerve retract to form the adult pattern. Similarly, in parts of the parasympathetic nervous system, each developing ganglionic neuron is initially innervated by several axons, each of which arises from a different neuron. In this system also, multiple innervation becomes single, or at least reduced, by elimination of some of the original afferents.

In some areas of the central nervous system, a similar phenomenon is observed. Many climbing fibers impinge upon a single cerebellar Purkinje cell in young brains, but only one fiber innervates the adult cell. An analogous but more complex arrangement exists in the visual cortex, where, in the adult, information from each retina dominates alternating bands of cortex in a pattern called ocular dominance columns. In the developing brain, however, retinal inputs overlap. If input from one eye is eliminated during a short critical period during development, the ocular dominance columns fail to develop. Thus, in the changing patterns of development, there can be many sites and times at which the developing nervous system is vulnerable to external stimuli.

L. SPEAR ("Drug-Vulnerable Events in Neuronal Development: Implications for the Selection of Test Methodologies") categorized the ways in which drugs influence development into nonspecific, general, and specific effects. External (stress, nutrition) and internal (hormonal or body temperature changes) stimuli, or drugs that alter these stimuli, can influence the development of the nervous system. A general effect on development can be produced by agents that will have quite specific effects later on. For example, drugs that alter monoamine neurotransmitter systems will have profound effects if given early in development, because monoamines play an important role in closing the neural tube and leading to the generation of neurons. Later, these drugs will only alter the specific system on which they act. An example is the action of neuroleptic drugs, which produce specific blockade of dopamine receptors but have different effects in the adult and fetus. In the adult, chronic haloperidol administration increases the response to dopamine agonists, whereas prenatally, the chronic administration of haloperidol decreases the response to dopamine agonists. Thus, several parameters of drug action may be different in the adult and fetus.

J. WEST ("Alcohol and Brain Development") addressed another set of factors that must be considered when examining the effects of drugs on the developing nervous system. When considering the possibility of morphological damage, one must be aware that both regional and temporal vulnerabilities may exist. These vulnerabilities will interact with both the dose of the administered drug and the pattern of administration. Thus, certain brain regions will be vulnerable only at certain times, if at all, and the same amount of drug that has no effect when administered over the course of a day may have a profound effect if given as a single dose. In addition, because different brain regions grow at different times in different animals, one must be careful to compare equivalent periods of development when examining drug effects in various species.

I. ZAGON ("Endogenous Opioids, Opioid Receptors, and Neuronal Development") examined the gross effects of endogenous opioids on various growth indices by administering an opiate antagonist, naltrexone, to rat pups. Body, brain, and organ weight, and the appearance of behavioral landmarks such as the righting reflex and walking, were employed in this study. Interestingly, if naltrexone was administered at a low dose of 1 mg/kg/day, there was a decrease in growth rate, but at a high dose of 50 mg/kg/day, there was an increase, as indicated by augmented body and organ weights. A low dose of 3 mg/kg/day given in three equal doses throughout the day, however, also produced a growth increment like that seen with the high dose. This suggests that the crucial requirement for faster development was constant occupation of opiate receptors by the antagonist, whether achieved by intermittent administration of low doses or by a single high dose. These findings illustrate the crucial role that dose and pattern of drug administration play in producing an effect.

C. KUHN ("Endocrine Consequences of Perinatal Methadone Exposure") described the effects of administering drugs chronically on the release of anterior pituitary hormones. Chronic methadone produced an increased release of growth hormone (GH) that persisted for 25 days of treatment. This persistent response to methadone differed from that to chronic pentobarbital or clonidine. The latter two drugs produced biphasic effects, first inhibiting GH release, then enhancing It, but at different times during chronic drug treatment.

Methadone had no effect on the secretion of luteinizing hormone (LH). Naloxone, however, increased its secretion, supporting the prevailing hypothesis that opioids control LH secretion by tonic inhibition of its secretion. The role of serotonin in the release of GH was investigated by administering cycloheptadene, an antagonist of serotonin, to neonates and adult rats. Cycloheptadene did not affect GH release following opiate administration in the neonate, but did alter the response to opiates in the adult. The conclusion is that the serotonin control of release has not yet developed in the neonate.

On the second day of the conference, plasticity in the adult nervous system was the topic of discussion. In contrast to the global types of effects seen during development, drugs administered to adults tend to have much more limited and specific effects, some of which are clearly reversible. The mechanisms of some of these effects were described by the second group of speakers.

D. JOHNSTON ("Cellular Mechanisms of Noradrenergic Enhancement of Long-Term Synaptic Potentiation in Hippocampus") described long-term potentiation (LTP). This important model for learning in the central nervous system is seen in the hippocampus, where a given response can be augmented by previous stimulation of the same pathway. Because this response facilitation can be graded, and because the underlying anatomical substrates are well described, this model is an important tool for the study of neuroplasticity. Norepinephrine (NE) and its second messenger cyclic AMP specifically enhance LTP, and, using patch clamp techniques, a calcium conductance in the neuronal membrane was also implicated in its generation. Because brain NE systems contain large numbers of opiate receptors, endogenous and exogenous opioids are strategically placed to alter profoundly the modulating effects of NE on hippocampal activity.

D. WALKER ("Neural Adaptation in the Hippocampus Induced by Long-Term Ethanol Exposure") described the effects of chronic alcohol administration in adult animals. Whereas all animals show a decrement in LTP following chronic alcohol administration, there may be genetic regulation of the vulnerability to some other effects of alcohol. Some animals, for example, show a greater loss of neurons in the hippocampus than others. Moreover, alcohol reduces the ability of the brain to regenerate following damage. This last finding illustrates the important point that many drugs

cause both destructive and compensatory changes. As a result, a functional deficit may be due more to the compensatory changes than the original damage. Because the relative importance of the destructive and compensatory changes will vary with time, it is important to distinguish between them when studying the effects of chronic drug administration.

P. GROVES ("Amphetamine Changes Neostriatal Morphology") described the normal ultrastructure of the striatum and how this is changed by amphetamine. After amphetamlne administration, dopamine terminals in the striatum become swollen and distended and are subsequently lost altogether. The terminals that are lost are those that were predominantly on the necks of dendritic spines, a particularly efficacious position on the spine from which to influence neuronal activity. This loss of terminals could explain why some effects of amphetamine weaken with repeated exposure. Interestingly, amphetamine also changed the shapes of dendritic spines, shortening their necks. Thus, terminals at the ends of the spines would be closer to the neuron and have greater potential to alter its activity. This finding could explain the potentiation (sensitization) of some other effects seen after repeated amphetamine exposure.

P. LANDFIELD ("Delta-9-Tetrahydrocannabinol-Dependent Alterations in Brain Structure") described the morphological changes that take place in the hippocampus due to normal aging and described how adrenal glucocorticoids play a key role in mediating these changes, which include cell loss. Because THC causes a large increase in glucocorticoid secretion, Landfield examined the effects of long-term THC administration on hippocampal structure. Rats chronically exposed to THC showed a loss of neurons and other changes indicative of neural damage. This study and the work of Dr. Groves, both of which used electron microscopic techniques, illustrate the importance of long-term studies relying on rigorous morphological analysis to examine the subtle effects produced by drugs.

M. CHRISTIE ("Mechanisms of Tolerance to Opiates in Locus Coeruleus Neurons") reviewed some of the problems that attend the electrophysiological study of drug effects when whole animals or tissue preparations are used. These include the possibility of multiple receptors, a response that is remote from the binding site, and the development of tolerance. By contrast, if one looks only at isolated membranes, there is no response, or the response that exists may not be coupled to the normal physiology of the organism. These considerations lead to the use of single neurons for many types of studies. Christie then described studies of opiate-tolerant locus coeruleus neurons. These studies indicate that the site of tolerance is in the coupling of the μ opiate receptor that exists on these neurons to the G(-) protein that controls the activity of adenyl cyclase. The cyclase produces cyclic AMP, an intracellular messenger whose level is ultimately responsible for the opiate effect.

J. DONOGHUE ("Cholinergic Modulation of Sensory Responses in Cerebral Cortex") described the importance of using intact animals and activating existing input systems to verify the effects of applied drugs. He reported that acetylcholine changed the response properties of neurons to tactile inputs. Thus, some neurons that were not spontaneously active and did not respond to tactile inputs under control conditions became active and had a vigorous response to tactile inputs after acetylcholine was applied to them. These kinds of neurons would normally have been overlooked in a study that only examined neurons with spontaneous activity, as Is commonly done.

R. DYKES ("Control of the Neuronal Receptive Field in Somatosensory Cortex") reported that acetylcholine, in addition to potentiating the responses of some cortical neurons to tactile inputs, may cause a prolonged increase in responsiveness in these neurons. Thus, not only is the response enhanced while acetylcholine is being applied, but it remains enhanced for many minutes after the drug has been removed or inactivated. This finding highlights the important caution that the effect of a drug may actually change even while it is being studied during acute administration.

In summary, the material presented in this volume makes clear that simple, straightforward analyses of drug action can only be accomplished with great caution. Studies of chronic drug exposure, especially in the developing organism, must take into account a wide variety of complicating factors when determining the actions of a drug. Some of these factors have been alluded to here and are described more fully in the chapters that follow. The growing awareness of the complexity of this kind of analysis represents an opportunity to enrich our understanding of how drugs act on the nervous system and to study, at a high level of resolution, the mechanisms by which their actions are mediated.

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External Factors Influencing Brain Development

Bruce S. McEwen

INTRODUCTION

In the history of biology, the "nature-nurture" controversy refers to the question of whether genetic constitution or environment holds the key to the development of the individual. For much of its history, this argument has occurred in the absence of knowledge about the essential question: What is the link between the environment and the genome? Nowhere is this issue more important and current than in the study of the brain.

For many years, the focus of neurobiology was on how nerve cells conduct and transmit electrical signals, and the brain was frequently modelled as a device which stores information like a computer. However, the influence of the environment on the nervous system, occurring through both sensory input and chemical signals, could not be ignored. For example, studies on environmental enrichment revealed structural changes in cortical thickness and cellularity produced by environmental manipulations, even in fully adult animals (Bennett et al. 1964; Greenough 1975). Moreover, investigations of the hereditary disease phenylketonuria revealed that diet, i.e., withholding phenylalanine, could control the onset and severity of the mental retardation caused by the disease (Brady 1976). Furthermore, studies of the influence of the gonads and adrenals revealed long-term effects of hormonal secretions on sexual and aggressive behaviors and sensory detection and recognition thresholds, as well as brain chemistry and structure (Leshner 1978; Henkin 1970; McEwen et al. 1979; Arnold and Gorski 1984). The connection between long-term influences of steroid hormones and the genome was further strengthened by the finding that steroid hormones bind to intracellular receptors which interact with the genome and affect gene expression (McEwen et al. 1979). Thus, whereas the controversy between "nature and nurture" placed genetic and environmental influences on two parallel lines and then argued about the relative magnitude of their respective effects (figure 1A), the current view of this Issue allows for a considerable plasticity in the ability of environmental influences to modify cellular biochemistry and morphology (figure 1B). In

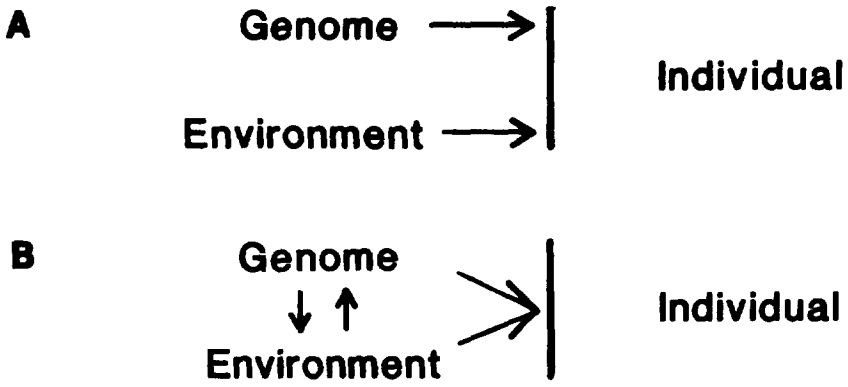

FIGURE 1. *Models of interaction between genome and environment in affecting individual traits*

this current view, the role of the genetic constitution of each individual is to set the limits for the effects of external agents on variable gene expression.

It is increasingly evident that the genome is in two-way communication with the cytoplasm and with the external environment. Steroid hormones affect cellular functions via the receptor-mediated activation and repression of genes. Other peptide and amino acid hormones and neurotransmitters can also influence the genome through second messenger systems. Both of these mechanisms are represented in figure 2. It is also apparent that steroids and other hormones interact with each other to produce their effects. On one hand, steroids regulate the ability of certain other hormones or neurotransmitters to act; on the other hand, neurotransmitters appear to influence the ability of steroid hormones to produce their effects upon brain cells (see figure 3). Insofar as drugs interact with either neurotransmitter receptors or steroid receptors, their long-term influences may be understood, at least in part, through the general scheme outlined above. This article addresses the issue of variable gene activity in brain cells as it is influenced by steroid hormones. These examples may help to provide insights into how drugs can produce or modulate nervous system function over a long time-course.

VARIABILITY OF GENE EXPRESSION THROUGH THE LIFE CYCLE

Background

Understanding how external factors such as hormones turn on genes is not the only important goal in relation to the interaction

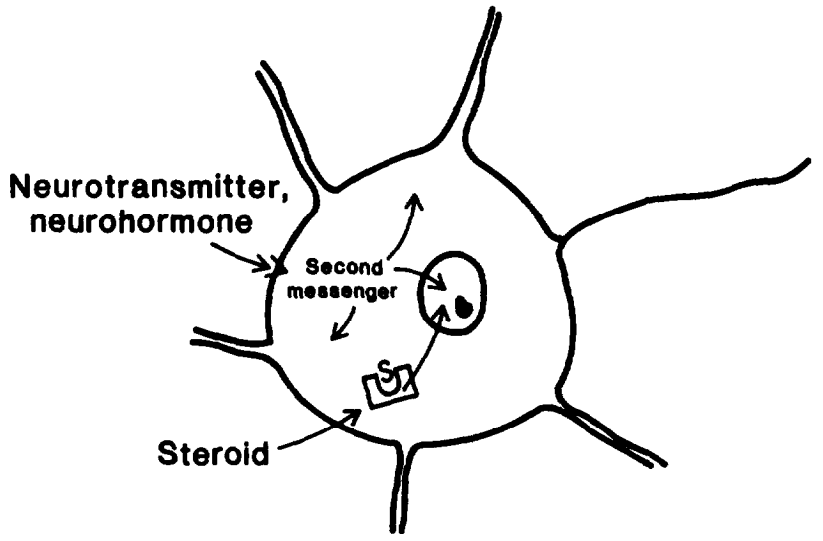

FIGURE 2. *Model of how steroid hormone and cell surfacs acting hormone may affect genomic activation*

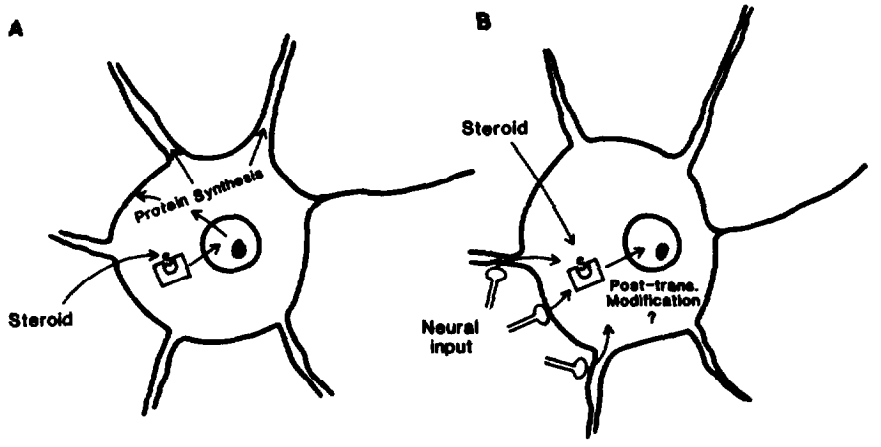

FIGURE 3. *Models of how neurotransmitters and steroid hormones interact with eaah other*

between the environment and the genome. Equally important is the fact that genes are turned on and off at various times during the life cycle, and the same chemical signals may have different effects at different stages of development and maturity. We now recognize five distinct categories of hormone actions during the life cycle. During early development, gonadal and adrenal steroids direct the differentiation and development of populations of neurons which have expressed intracellular steroid receptors as part of their differentiation. In mature nerve cells, on the other hand, circulating hormones mediate cyclic and behaviorally induced changes in function which are largely reversible, even though they may last for many hours. In contrast, in the aging organism, steroid hormones of adrenals and gonads play a role in promoting neural damage and neuronal loss. Such deleterious influences also occur with prolonged exposure to hormone at virtually any stage of development. Surprisingly, however, hormones are also instrumental in promoting plastic changes in damaged neural tissue which result in replacement of lost synaptic connections. In such cases, the hormone appears to act on developmental programs which are reactivated by the damage. Let us briefly consider some examples of these five categories.

Developmental Effects

During perinatal development in rats and prenatal development in guinea pigs and primates, testosterone secretion in the male fetus leads to sexual differentiation of the reproductive tract and the brain (Goy and McEwen 1980). These effects are mediated by androgen and estrogen receptors that are laid down in both sexes during embryonic life, and it is the secretion of testosterone only in the male during a sensitive or "critical" period of development which is responsible for the male's becoming different from the female. Testosterone is actually a prohormone: its metabolftes, estradiol (E) and 5α dihydrotestosterone (DHT), produced locally in target cells, are the actual agents which bring about sexual differentiation. Thus E, normally thought to be a "female" hormone, is partially responsible for the masculfnizatfon (enhancement of male traits) and defeminization (suppression of female traits) of the brain. E and DHT are believed to produce some of their principal effects by inducing neurite outgrowth and promoting neuronal survival (Toran-Allerand 1984; Arnold and Gorski 1984). In dofng thfs, E and DHT are undoubtedly acting in concert with other morphogenetic substances such as the various nerve growth factors, and some of these possible interactions will be described below. What results from the actions of E and DHT during the critical period is a nervous system in which discrete neuroanatomical loci have undergone morphological and biochemical differentiation in the two sexes. Brain sexual differentiation is perhaps the clearest and most important example of the developmental effects of hormones, not only because of the insights thus far obtained into cellular mechanisms but also because of the implications which a sexually differentiated neural substrate has for adult neural functions.

Cyclic Activation of Neuronal Characteristics

After early development, when neuron populations are reduced to adult numbers and stable synapses are formed, hormones have somewhat different effects on neuronal biochemistry and function which are largely reversible. The gonadal and adrenal hormones are each secreted cyclically (diurnally, monthly, or seasonally), and this produces cyclic effects on neuronal activity and biochemistry. One example is the synaptic efficacy for long-term potentiation (LTP) within the hippocampus, which varies with the time of day and peaks during the waking period following prewaking elevation of glucocorticoids (McEwen and Brinton, in press). Adrenalectomy causes this rhythm to shift 12 hours to peak during the sleeping period (Dana and Martinez 1984). Another example of a cyclic hormone effect is the sexual behavior of the female rat, which varies cyclically during the estrus cycle. The cyclic elevation of E followed by progesterone (P) is responsible for inducing sexual receptivity in female rats, and E and P do this by acting on a specific group of neurons in the ventrolateral/ventromedial nuclei (VMN) of the hypothalamus and inducing changes in genomic activity involving multiple gene products associated with neurotransmission (McEwen et al., in press a). An important connection to sexual differentiation exists, because male rats fail to show lordosis behavior when castrated and primed with E and P, even though it can be demonstrated that the female sexual reflex (called lordosis) is present in males. Males castrated at birth (thus escaping the postnatal "defeminization") show female receptivity when primed with E and P as adults. The "defeminizing" actions of testosterone (T) durfng the critical period involve the generation of E as the active metabolite (McEwen et al., in press b). Thus, the actions of E on the developing genome of the VMN have consequences which prevent E from acting on the same adult neurons to activate lordosis behavior in adult males. This example of differential actions of the same hormone in immature and adult neurons further emphasizes the point made above regarding the shifts in gene expression with development.

Behaviorally Induced Adaptive Responses

Another facet of reversible hormone effects in the mature nervous system involves the responses elicited by hormones secreted as a consequence of behavioral events. For example, aggressive encounters between males in defendfng territory prior to mating season elevate T levels, then T activates neural and reproductive tract systems, leading to successful mating and reproduction. This type of behavioral control results in much the same consequences as those produced by cyclic changes in hormone levels. In fact, in the realm of reproduction, the seasonal and behavioral regulatfon of hormone secretion are causally linked and, thus, not really different from each other. However, a form of behaviorally induced hormone secretion that is distinctly different from cyclic hormone release is the phenomenon of stress. Stress encompasses a variety of behavioral states or situations that induce a massive outpouring of CRF and vasopressin from the hypothalamus, ACTH and

ß-endorphin from the pituitary, and glucocortfcoids and epinephrine from the adrenals. The immediate impact of stress-induced hormone secretion appears to activate restorative and protective mechanisms in relation to emergencies. When stress is recurrent, the stress hormones help to bring about "adaptive" changes in the nervous system. One example of this is the stress-induced suppression of noradrener fc-stimulated generation of the second messenger, cyclic AMP McEwen and Brfnton, in press). This suppression occurs only after repeated stress and involves, in the cerebral cortex, a glucocorticoid-mediated suppression of the a-adrenergic contribution to cAMP generation (Stone et al. 1986). The fact that such a mechanism exists illustrates the specialization of means by which environmental signals of diverse types, intensities, and durations can bring about situation-specific changes in the central nervous system (CNS).

Degenerative Consequences of Hormone Action

When stress or glucocorticoid exposure is prolonged, the normally adaptive processes may give way to deleterious effects. Outside of the nervous system, these include fmmunosuppression and enhanced susceptibility to infectious agents and tumors; osteoporosis; muscle wasting; and steroid diabetes. The classic example of wasting away from too much glucocorticoid is the migrating salmon, which dfes after a crisis of elevated glucocorticoids brou ht on by the stress of migration upstream (Robertson et al. 1961). In a less dramatic way, glucocorticoids contribute significantly to loss of neurons in the hippocampus with age in the rat. This loss of neurons, together with a reduction in glucocorticoid receptors, leads to progressively elevated glucocorticoid levels because of lessening negative feedback capability (Sapolsky et al. 1986). It is conceivable that the same "feed-forward cascade" may be instigated by persfstent stress in younger individuals. and, in this connection, one may speculate that individual differences in stress susceptibility that are developmentally determined can predispose individuals to faster or slower entry into such a "feed-forward cascade." Indeed, neonatal handling of rats produces higher than normal glucocorticoid levels in the hippocampus and a better than normal capacity to shut off glucocorticoid secretion after stress (Meaney et al. 1985). It remains to be seen whether such early experience has the expected effect of retarding the aging process. Although incomplete, thfs example serves to illustrate the insights we are now beginning to have into the impact of the environment on the developfng nervous system and how it responds to the environment after it is mature.

Repair of Neural Damage

Strange as it may seem after the discussion of damage produced by steroids, the hormones of the gonads and the adrenals also have the capability of promoting new synapse formation. For example, homotypical sprouting of serotonfn nerve fibers in the hippocampus after damage of other serotonin projections results in reinnervation of vacant synaptic sites; this repair process is markedly

attenuated by bilateral adrenalectomy and restored by glucocorticoid administration (Zhou and Azmitia 1985). Furthermore, in the hypothalamus, cutting of major inputs to the arcuate nucleus results in marked loss of synapses; collateral sprouting and reinnervation of vacant synaptic sites is markedly facilitated by estrogen treatment (Matsumoto and Arai 1979). In both of these examples, it can be argued that developmental programs which were active in the embryo are once again called into play by the damage. Here is another example of the varfability of gene expression in the nervous system and how actions of hormonal sfgnals are dependent on the state of the genome.

INTERACTIONS BETWEEN NEUROTRANSMITTERS AND HORMONES

In addition to being dependent on the state of dfferentfatfon of the target cell, steroid hormone action is also dependent to some extent on other chemical messengers such as neurotransmitters. In development, such effects have been inferred on the basis of pharmacological studies in which neurotransmitter drugs are administered together with testosterone (T) during the critical period for sexual differentiation. The rationale for supposing that developmental effects of T may be dependent on neurotransmitters is that monoamine neurotransmftters are implicated in a wide range of developmental events in the nervous system (Lauder 1983). Indeed, administration of reserpine and phenoxybenzamine, drugs which deplete or block actions of monoamines, respectively, inhibits the developmental actions of T (Gorski 1973; Nishizuka 1976).

With respect to catecholamfnes, tyramine (which releases noradrenaline from terminals) as well as the receptor blockers phenoxybenzamfne and phentolamine inhibit the defeminfzing actions of T to suppress ovulatory cyclfcity in female rats (Raum and Swerdloff 1981). In these experiments, the fact that the ß blocker propranolol reversed the blockade of defemfnfzation led these authors to propose that noradrenaline suppresses masculinfzation via ß receptors, and presynaptfc a receptor blockade facilitates thfs mechanism (Raum and Swerdloff 1981). A subsequent study, using other drugs, provided evidence partially confirming this hypothesis, showing that ß receptors appear to mediate inhibition of T action, but the α--2-blocking drug yohimbine has no effect on T action (Vidal anđ Aguilar 1985). A plausible mechanism for ß-receptor-mediated inhibition of T action is the reported ß-receptor-mediated inhibition of the conversion of T to estradiol, which underlies defeminization (Vaccaro et al. 1980).

Serotonin is also implicated in developmental actions of T, and most of the evidence suggests that, in contrast to noradrenaline, serotonin may be involved in promoting the defeminftfng actions of T. For example, postnatal treatment of female rats with parachlorophenylalanfne (pCPA) protects agafnst the defeminizing actions of T on anovulatory sterility (Reznikov et al. 1979). In the absence of T, pCPA does not affect cyclicity, but it does reduce the amount of spontaneous proceptive behavior in females (Hyyppa et al. 1972). Conversely, tryptophan administration

postnatally potentiates T actions to suppress lordosis behavior, although in this study pCPA did not interfere with T-induced defeminization (Jarzab and Dohler 1984). Not all the evidence implies a protestosterone effect of serotonin, however. For example, 5-hydroxytryptophan administration delays anovulatory sterility induced by T (Shirama et al. 1975). and prenatal treatment with pCPA actually increases the volume of the androgen-dependent sexually dimorphic nucleus of the preoptfc area in female rats without altering circulating hormone levels (Handa et al. 1986). Moreover, forebrain levels of serotonin are higher in female rats than males at the end of the second postnatal week of life, and T administration to females or castration of males reverses the sex differences (Ladosky and Gaziri 1972; Giulian et al. 1973). Thus, serotonin may have diverse effects on brain development and sexual maturation which are brain region specific and also dependent on the time of postnatal life.

It is our view that global treatments of rats with drugs affecting the whole brain may not give sufficient information pertaining to neurotransmitter control of specific behaviors, and we have been exploring region-specific aspects of serotonin action. Since the basal hypothalamus is involved in control of lordosfs behavior in the female rat, we have concentrated our efforts on serotonin function in this brain region. Lesions of the serotonin innervation of the basal hypothalamus with 5,7 dihydroxytryptamine produce facilitation of lordosis behavior in female rats iven estradiol (E) plus progesterone (P) (Luine et al. 1983). Male rats with the same lesion show facilitation of lordosis behavior from a baseline which is close to zero; furthermore, 5,7 dihydroxytryptamine lesions unmask sensitivity to P which is not evident in unlesioned males (Moreines et al., unpublished). This unexpected result implies that serotonin plays an important role in the sexual differentfatfon of the male brain and is responsible, in part, for the lack of display of the lordosis reflex after E plus P priming. These results are consistent with the data, summarized above, which show that serotonfn potentiates the defeminizing aspect of T action, but it is also possible that serotonin is not directly involved in T action, but rather that T action increases the outgrowth of serotonin fibers into the basal hypothalamus and/or changes the regulatory effects of E plus P treatment which normally remove serotonin inhibitory influences and allow lordosis to be displayed.

The effects of serotonin to inhibit the actions of E and P in adult female and male rats imply that there may be other interactions between the neurotransmftter and the hormone. What are they? One type of interaction is the ability of E and P to alter monoamine oxidase activity and thus to change the turnover of monoamines such as serotonin (Luine and Rhodes 1983). Another type of interaction may be at the level of the E and P receptors themselves. Although evidence is lacking for serotonin, other monoamine neurotransmitters are implicated in influencing the level or availability of E and P receptors, For example, dopaminergic stimulation increases estrogen receptor numbers in the

pituitary, overcoming the effects of disconnection from hypothalamic input (Carrillo et al. 1983; DeNicola et al. 1981; Weisenberg et al. 1979). Stimulation of dopamfnergic receptors by bromcryptine also results in increased cell nuclear uptake of ^{3}H-E in vivo in the hypothalamus and the pituitary, an effect which is evident in female rats but not in males (Thompson et al. 1983; Gietzen et al. 1983; Gietten and Woolley 1986). In a related study, inhibition of dopamine-ß-hydroxylase results in increased numbers of hypothalamic nuclear estrogen receptors even in the absence of endogenous E, implying a conversion of E receptors to a nuclear binding form (Blaustein 1986). The opposite effect, a decrease in the number of nuclear E receptors in the hypothalamus, is reported in guinea pigs following a-l blockade by prazosin (Clark et al. 1985). Finally, prazosin or other a-receptor-blocking drugs reduce progestin receptor levels in the E-primed guinea pig hypothalamus, an effect which is restricted to the ventromedfal nucleus region that controls lordosis behavior (Nock et al. 1981; Nock and Feder 1984; Thornton et al. 1986). a-l Receptor blockade also inhibits the display of lordosis behavior under the same conditions, implying that there may be a causal connection (Nock and Feder 1984). It remains to be established whether serotonin may have sfmilar effects on E and P receptors.

The interaction between neurotransmitters and steroid hormones, outlined above, is represented in figure 3. In this figure, steroid hormones can induce specific protein gene products which may include enzymes and receptors involved in neurotransmitter action (panel A); alternatively, transmitters may modify steroid hormone action and may do so by modifying the hormone receptors themselves or the ways in which they produce their gene regulatory effects (panel B).

CONCLUSIONS

This paper began with the thesis that long-term effects of drugs on the brafn involve changes in genomic activity, and we have illustrated the general topic of environmental influences on the genome by examples pertaining to adrenal and gonadal hormone effects on the brain. We have seen that steroid effects involving the genome are diverse, and this diversity is the product not only of the heterogeneity of the target brain cells but also of their state of differentiation. In other words, the same hormonal signal can influence target cells in different ways depending on which sets of genes are available to be acted on by the hormone-receptor complex. By analogy with steroid hormones, we know that many neurotransmitters and surface-acting hormones can also influence genomfc activity via second messenger systems which alter phosphorylation of proteins that interact with genes (figure 2).

If we extrapolate from hormones to neuroactive drugs, we can make a number of general observations about the mechanism of long-term drug effects. First, the diversity of drug actions in development, adult life, aging, and after brain damage may be similarly based on the state of differentiation of target cell genomes at

the time of drug administration. Secondly, drugs may act in part by modifying the secretion of hormones, thus producing long-term effects via the actions of these hormones. Thirdly, drugs which act via neurotransmitter systems may modify the actions of hormones which are acting on the same cells. Finally, it is conceivable in a few instances that psychoactive drugs may actually mimic hormones by interacting directly with hormone receptors. Such interaction with estrogen receptors has been reported for a number of classes of psychoactive drugs, including phenothfazfnes such as chlorpromazine, ergot alkaloids such as bromcryptine, digftoxin and reserpine (Shani et al. 1971; Campbell and Clark 1984; Liel et al. 1982; Levy et al. 1980).

In the case of two drugs of current interest, alcohol and narcotics, it is possible to point to long-term effects on aspects of sexual development. In rats, prenatal alcohol exposure has been shown to abolish sex differences in saccharin preference and Lashley III maze performance, even though it does not evidently masculinize or feminize reproductive behavior (McGivern et al. 1984). The mechanism for this rather selective effect is therefore unlikely to involve wholesale alterations in gonadal hormone secretion or actions but rather more selective effects on neural development. In contrast to alcohol, morphine has been reported to affect reproductive behavior in rats when administered pre- and postnatally. Prenatally, morphine treatment results in precocious vaginal opening and inhibition of feminine sexual behavior; male offspring, on the other hand, showed minimal impairment of reproductive behavior (Vathy et al. 1985). Postnatal treatment of rat pups with morphine produced the opposite effect to prenatal treatment; namely, enhanced feminine sexual receptivity and less inhibition of receptivity by acutely administered morphine in adulthood (Meyerson and Berg 1985). Because there are no major aberrations in other aspects of sexual development, it appears that major effects of morphine on testosterone action and/or secretion are unlikely explanations. Rather, more direct influences of morphine on neural development are suggested (Zagon, this volume).

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Rearrangement of Synapses During Development

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INTRODUCTION

During the development of the nervous system, neurons and their targets establish appropriate synaptic connections in two respects. First, each nerve cell innervates particular postsynaptic cells, avoiding other potential synaptic partners which are in some sense qualitatively incorrect. Second, appropriate neural connectivity depends on precise regulation of the numbers of connections established: each axon must innervate an appropriate number of target cells, and each target cell must be innervated by an appropriate number of axons. The regulation of quantitative aspects (convergence and divergence) is based on competitive interactions, the outcomes of which are not preordained. Because of this, these interactions are exquisitely sensitive to environmental stimuli. The aim of this paper is to describe a variety of systems which provide information about how quantitative aspects of innervation are regulated. It seems likely that a relatively small number of principles may underlie the diversity of the patterns of innervation found in the nervous system, the most salient of which is the importance of competitive interactions in establishing the immutable pattern of connections found in maturity.

SYNAPTIC MALLEABILITY AT THE DEVELOPING NEUROUUSCULAR JUNCTION

In the early part of this century, neuroanatomists noted the different appearance of silver-stained neuromuscular junctions in neonatal and mature muscles (Boeke 1932; Tello 1917). They found that, whereas adult mammalian muscle fibers are contacted by a single axon terminal, several different axons converge at the endplate of each neonatal fiber. More recently, Redfern (1970) showed by electrophysiological means that skeletal muscle fibers in neonatal animals are in fact innervated differently from adult cells. Intracellular recordings from individual muscle fibers in neonatal animals showed multiple steps in the postsynaptic potential elicited by gradually increasing the strength of motor nerve stimulation. In adult animals, only one step was seen. These results have been confirmed in many vertebrate muscles. They

provide evidence for transient innervation by several different axons at the same muscle fiber (Bennett and Pettigrew 1974; Brown et al. 1976). Furthermore, morphological, electrophysiological, and pharmacological studies imply that all the initial synaptic contacts (each fiber receives synaptic input from at least two to three axons) are located at approximately the same place along the fiber length; namely, the eventual site of the mature endplate (Bennett and Pettigrew 1974; Bennett and Pettigrew 1976; Brown et al. 1976; Korneliussen and Jansen 1976; Riley 1976; Riley 1977a; Riley 1977b; Riley 1981).

Because the transition from the neonatal to the adult pattern of muscle innervation occurs by the removal of some initial neuromuscular synapses, this process is called synapse elimination. For technical reasons, the time of onset of elimination has been difficult to assess. In some mammalian muscles, it certainly begins before birth (Dennis et al. 1981) but continues into the first few weeks of postnatal life. On the other hand, in some reptilian muscles, neuromuscular synapse elimination is complete by birth (Lichtman et al. 1985). Because there is little evidence of nerve terminal degeneration in neonatal muscles (Bixby 1981; Korneliussen and Jansen 1976; although see Rosenthal and Taraskevich 1977), it seems likely that the terminals which are lost are withdrawn into the parent axon. Indeed, profiles of what appear to be retracting processes are often observed in neonatal muscle (Bixby 1981; Riley 1981). New techniques in which the terminals of different axons can be labelled different colors suggest that the transition from multiple to single innervation occurs by the gradual occupation of the endplate site by one axon as the other axons gradually lose their synaptic contact (Lichtman, unpublished).

Synapse elimination generally occurs without an obvious change in the number of innervating motor neurons or target fibers (Bagust et al. 1973; Brown et al. 1976; Oppenheim and Majors-Willard 1978; see, however, Nurcombe et al. 1981). This means that the number of muscle fibers contacted by each motor axon (the motor unit) must be sharply limited during early postnatal life. Motor unit size can be measured by estimating the percentage of total tension in a muscle that is developed by each axon; such measurements show that there is a several-fold decrease in the average size of motor units during the first few weeks of life. The largest motor units tend to undergo more elimination than smaller ones.

Most studies of synapse elimination have looked at skeletal muscle fibers that are ultimately innervated at a single point along their length; a few studies have also examined synapse elimination from muscles that are multiply innervated in maturity. Such multiply innervated muscle fibers in vertebrates are usually contacted at more or less regular intervals by a number of different synaptic endings (Wilkinson and Lichtman 1985). These muscle fibers are slowly contracting (tonic) in which there are no propagated action potentials. Each endplate site along such fibers in a snake muscle is contacted by several axon terminals early in

life, and, interestingly, multiple innervation is maintained (Lichtman et al. 1985). The inability of the target cells to fire action potentials may be the reason for the absence of synapse elimination in tonic fibers (see below).

SYNAPTIC REARRANGEMENT IN THE PERIPHERAL AUTONOMIC NERVOUS SYSTEN

A similar reduction in the number of axonal inputs to target cells occurs at the synaptic junction between neurons. Probably the simplest neuronal system in which synapse elimination has been studied is the submandibular ganglion of the rat (Lichtman 1977; Lichtman 1980). Cells in this parasympathetic ganglion are similar to skeletal muscle fibers in that most of them are strongly innervated by a single axon in maturity. As in muscle, these ganglion cells at birth are innervated quite differently: each neuron is initially contacted by about five different preganglionic axons. A further similarity to mammalian muscle is that the initial convergence of several different axons onto each target cell is reduced to the adult pattern over the first few weeks of postnatal life. Finally, in another parasympathetic ganglion, it is clear that the innervating population of preganglionic neurons remains constant during the period of synapse elimination (Johnson and Purves 1981). Thus, neural units are decreasing in size at the same time as motor units are decreasing.

The remarkable congruence of synaptic rearrangement in muscle and simple parasympathetic ganglia raises the question of whether the phenomenon of synapse elimination is a special strategy restricted to those muscular or neural targets in which there is ultimately a need for a one-on-one pattern of innervation. However, some classes of neurons that remain multiolv innervated in maturitv also undergo some degree of synaptic rearrangement in early life. In the hamster suoerior cervical ganglion, for instance. in which each adult neuron remains innervated by a number of different axons (about seven), neonatal ganglion cells are innervated by about twice as many different axons (Lichtman and Purves 1980). Interestingly, however, the vigor of the elimination process seems less in ganglia in which neurons remain multiply innervated.

At first glance, one feature of synaptic rearrangement in autonomic ganglia appears different from that in muscle: the number of identifiable synapses actually increases during the period when syna ses are being eliminated (Lichtman 1977; Johnson and Purves 1981p. In fact, a gradual accumulation of synapses during early postnatal life appears to be characteristic of many parts of the nervous system (see Purves and Lichtman 1985a for review). Because new synapses more than compensate for the numerical loss of synapses during this period, the phrase "synapse elimination" obscures the important features that synaptic rearrangements in muscle and autonomic ganglia have in common: a reduction in the number of target cells contacted by an axon and a reduction in the number of axons contacting each target cell. Thus, it appears that each innervating axon initially establishes a relatively small number of synapses on a relatively large number of target

cells. As development proceeds, these initial synaptic contacts are redistributed and additional synapses are formed, so that more and more synaptic endings are established by each axon on fewer and fewer postsynaptic cells. The essence of this process, then, is a focusing of innervation rather than elimination.

On the face of it, the innervation of muscle provides an exception to this rule: if each mature muscle fiber is innervated by a single synapse in maturity, and several different axons innervate each fiber at birth, surely there must be a net loss of synapses. Although referred to as a single synapse, the vertebrate neuromuscular junction actually comprises a number of individual boutons. Since the size and complexity of the boutons clustered within an endplate increase postnatally (Davey and Bennett 1982; Nystrom 1968), increasing numbers of synaptic boutons are probably elaborated on muscle fibers, just as on the surface of autonomic ganglion cells (and other neurons) during this period. Thus, in muscle as well, the synaptic connections from the remaining axon become progressively larger as the number of cells they remain in contact with declines.

It is also important to point out that, in all the systems studied, it is very unlikely that the purpose of synapse elimination is to remove erroneous connections. The earliest connections formed between presynaptic neurons and vertebrate muscle fibers or ganglion cells are largely correct. Thus, relatively few errors are ever present (Landmesser 1980; Lichtman and Purves 1980; Rubin 1984). Moreover, during synaptic rearrangement an axon loses its contacts with some target cells but retains contacts with other nearby cells whose function seems the same; it is difficult to see how one set of connections in a muscle or functionally homogeneous parasympathetic ganglion is in some qualitative sense more appropriate than any other. On the other hand, synapse elimination in some autonomic ganglia is weakly influenced by the segmental origin of the innervating axons (Lichtman and Purves 1980). A similar influence during muscle innervation would not be surprising given the fact that cholinergic axons show a weak se mental preference in muscle innervation (Wigston and Sanes 1982) . Indeed, some evidence for selective synapse elimination has been presented (Miyata and Yoshioka 1980; see, however, Gordon and Van Essen 1983; Thompson 1983b). It is probably true that, to the extent that qualitative criteria influence synaptogenesis, they will also influence synaptic elimination.

SYNAPTIC REARRANGMENT IN THE CENTRAL NERVOUS SYSTEM

Similar phenomena have been described in several parts of the central nervous system (CNS). For example, in the avian auditory system, neurons in the nucleus magnocellularis are also innervated by more axons before birth than after (Jackson and Parks 1982). As in the peripheral nervous system, the decline in axonal convergence is accompanied by a decrease in the number of terminal branches arising from the innervating axons. The innervation of Purkinje cells by climbing fibers in the cerebellum of newborn

rats undergoes a similar rearrangement. Whereas multiple climbing fibers innervate each Purkinje cell in neonatal rats, in mature animals each Purkinje cell is contacted by only a single climbing fiber (Crepe1 et al. 1977).

On the other hand, not all synaptic connections in the CNS are susceptible to elimination. The connections from stretch sensitive sensory afferents (Ia) to muscle fibers do not seem to undergo elimination. This is inferred from the fact that no sensory connections that are not also present in adults are found in development (Frank and Westerfield 1983), and, in the adult, virtually every appropriate stretch sensitive axon innervates every appropriate motor neuron, meaning that none of these appropriate connections were eliminated either (Lichtman and Frank 1984). In contrast to synapses where synapse elimination has been documented, the Ia connections are quite weak; no single Ia axon can drive a motor neuron to threshold.

The best studied and most informative example of rearrangement of initial innervation in the CNS is the developing visual cortex. In adult cats and in some species of monkey, neurons in the primary visual cortex are segregated into columns dominated alternately by the right and left eye (Hubel and Wiesel 1965; Hubel et al. 1977; LeVay et al. 1978; Rakic 1977; Shatz and Stryker 1978; Wiesel and Hubel 1962; Wiesel and Hubel 1965). In contrast to the adult, at birth (and in late embryonic life) there is considerable overlap of adjacent columns from the right and left eye (Hubel et al. 1977; Rakic 1977). Thus, initially the arborfzations of geniculate axons driven by each eye overlap in layer IV of the primary visual cortex. The outcome of this rearrangement is very sensitive to neural activity (see below).

Similar segregative phenomena occur in other cortical and subcortical areas. For instance, inputs are gradually segregated into upper and lower cortical layers in the olfactory cortex of developing mammals (Schwab and Price 1984). In the lateral geniculate nucleus, retinal afferents from both eyes are initially distributed throughout the nucleus but are eventually restricted to particular laminae (Rakic 1977; Sretavan and Shatz 1984; Sretavan and Shatz 1986).

Under experimental conditions, segregation can even be induced between afferents that would not normally encounter one another. Thus, if optic nerve fibers from two eyes of lower vertebrates are forced to innervate the same side of the tectum, the two sets of inputs tend to segregate into discrete patches (Constantine-Paton and Law 1978; Law and Constantine-Paton 1981; Law and Constantine-Paton 1982; Fawcett and Willshaw 1982).

Other rearrangements have been described in the CNS which are less clearly related to the sorts of rearrangements of synaptic connections that have been studied in the peripheral nervous system and the primary visual system. For example, many axons extend across the corpus callosum to the contralateral hemisphere in mammals.

Studies using retrograde and anterograde tracer methods show that these crossing projections are more diffuse in young animals than in adults (Innocenti 1981; Innocenti 1982; Innocenti et al. 1977; Ivy and Killackey 1981; Ivy and Killackey 1982; Ivy et al. 1979). Many of the neurons that lose their interhemisphere connections survive into maturity; therefore, this restriction is the result of terminal retraction rather than cell death (Innocenti 1981; O'Leary et al. 1981; Stanfield et al. 1982). Whether synapses are eliminated during these retractions is not known.

FACTORS REGULATING SYNAPTIC REARRANGMENT

The final result of synaptic rearrangement during development is very different for different types of cells. As already mentioned, some target cells are ultimately innervated by a single axon, as is the case for the motor neuron input to twitch muscle fibers, some autonomic ganglion cells, and the climbing fiber input to Purkinje cells, whereas other cells are innervated by literally thousands of axons, as is the case for many classes of cells within the CNS. This wide range of convergence raises a fundamental question: Why does synapse elimination sometimes continue until only one axon remains in contact with a target cell, whereas in other instances multiple innervation persists?

In autonomic ganglia, an obvious difference between cells that are singly innervated and neurons that remain multiply innervated is their geometry. Thus, mature neurons in the submandibular ganglion (which are singly innervated) lack dendrites altogether, whereas neurons in the mammalian superior cervical ganglion (which are multiply innervated) have a number of different dendrites whose number is roughly proportional to the average number of ganglion cell inputs (Purves and Lichtman 1985b).

The relation between target cell shape and number of inputs has been explored directly in the rabbit ciliary ganglion in which the cells have more or less the same function and display both a range of geometries and a range of inputs (Johnson and Purves 1981; Johnson and Purves 1983). Cilfary ganglion cells that are innervated in maturity by a single axon usually lack dendrites altogether, as do submandibular ganglion cells. Conversely, ciliary ganglion cells innervated in maturity by more than one axon have geometries that on average increase in complexity in roportion to the number of innervating axons (Purves and Hume 1981p. The adult geometry of ganglion cells does not appear to be a result of the number of axons which initially contact each cell. All rapid ciliary ganglion cells are multiply innervated at birth. Yet the geometry of ciliary ganglion cells at birth shows the same range of complexity as the adult: some cells lack dendrites altogether, other cells have modest dendritic arborizations. and still others have arborizations as complex as the dendritic trees in mature ganglia (Hume and Purves 1981). Apparently, the confinement of an initial set of inputs to a limited postsynaptic surface (the cell body) forces a resolution in which synaptic contacts from only one axon can survive. Conversely, the presence of dendrites in an

early stage somehow confers immunity from these interactions between axons, so that innervation by a number of different axons persists. Immunity from synapse elimination might be conferred because the presence of dendrites allows inputs from different axons to become segregated to some degree (Forehand and Purves 1984). On the other hand, synapses from different axons persist side by side on ciliary ganglion cells (Forehand, submitted for publication). Thus, dendrites might mitigate elimination in other ways, perhaps analogous to the way in which tonic fibers maintain multiple innervation, because in both cases the target membrane is inexcitable (see below).

COMPETITIVE ASPECTS OF SYNAPTIC REARRANGEMENT

Competition between different axons innervating the same target cells seems to be an important feature of the process of synaptic rearrangement: terminals are withdrawn from a target cell because in some sense other terminals have been more successful. Several lines of evidence support this view. When motor unit size is measured in very small muscles in which only a single axon has been left to innervate the entire muscle, the remaining motor unit fails to decrease in size (Betz et al. 1980). In some developing muscles, there is also evidence for an intrinsic tendency for axons to withdraw a portion of their initial arborization. For instance, if a substantial fraction of the innervation to rat soleus muscle is removed at birth, in this muscle an appreciable reduction in motor unit size still occurs (Brown et al. 1976). This may occur because axons are, as already described, in fact redistributing (rather than eliminating) synaptic "machinery" which may be limited in total amount. A further argument for competition derives from the end result of rearrangement in muscle and some autonomic ganglia. It is difficult to imagine how a process that did not involve competition could produce the one-on-one relationship observed between these target cells and their innervation. If the withdrawal of axons were simply random, then some target cells would be expected to receive no innervation, whereas others should end up with more than one axon supplying them. A further indirect argument for competition is the push-pull nature of the rearrangement that takes place in the peripheral nervous system: labelling of motor nerve terminals shows that, as one axon increases its hold on the postsynaptic cells, others apparently let go (Lichtman, unpublished).

If competition is a central feature of synaptic rearrangement, what do axons compete for? One possibility is trophic factors produced by the target cells. Trophic interactions between target cells and the neurons that innervate them is a basic feature of neural development. Thus, the death of many neurons in early embryonic life apparently results from failure to obtain trophic support from peripheral targets or, in some cases, to provide it (Oppenheim 1981). In at least two parts of the nervous system, there is good evidence that this trophic support is provided by a specific protein (nerve growth factor) which may be produced by target cells (Levi-Montalcini and Angeletti 1968; Thoenen et al.

1982). It is attractive to suppose that axons also compete for target-produced factors during the synaptic rearrangements. In this view, the number of target cells innervated by each axon is regulated in large measure by the presynaptic cells' requirement for a particular level of trophic support. Since axons gradually reorganize their initial connections to innervate a smaller number of target cells, an axon presumably receives more trophic support by innervating a few cells strongly than many cells weakly.

NEURAL ACTIVITY'S ROLE IN SYNAPTIC REARRANGEMENTS

The scenario in which target cells supply limited amounts of trophic molecules to competing presynaptic cells does not entirely account for what is observed. For example, the number of synaptic boutons actually increases during the period of interaxonal competition in early life. Therefore, trophfc support must be available for a much larger number of synaptic connections than is initially present; what seems to be lacking is support for connections arising from many different axons contacting the same target cell. Moreover, the idea of competition for a limited supply of trophic factor in the local environment of nerve terminals does not explain the striking capture of individual cells in both the neuromuscular and autonomic nervous systems. If presynaptic axons simply required a threshold level of trophic factor to be maintained in a particular region, there would be no reason for the sharp focusing of innervation on a few target cells during synaptic rearrangement.

These considerations all suggest that the synapses which ultimately innervate a muscle cell or neuron are somehow treated as "sets" during the process of innervation. Evidently, all the synaptic boutons arising from one axon are identified during competition as being different from the synaptic boutons arising from a competing axon. How is this identity of sets accomplished? An obvious property held in common by synaptic boutons arising from a particular axon is their pattern of neural activity. There is, in fact, considerable evidence that synaptic rearrangements are influenced by activity. For example, in both muscle and autonomic ganglia, chronically paralyzing nerves with a local anesthetic (or otherwise decreasing activity) decreases the rate of synapse elimination (Benoit and Changeux 1975; Benoit and Changeux 1978; Caldwell and Ridge 1983; Jackson 1983; Riley 1978; Srihari and Vrbova 1978; Thompson et al. 1979). Conversely, increasing the level of activity of innervating axons by chronic stimulation speeds up elimination (O'Brien et al. 1978; Thompson 1983a). Additional evidence for the influence of neural activity on synaptic rearrangement comes from the development of the visual system. If one eye of a monkey is occluded during the first few weeks of life, the normal segregation of ocular dominance columns is dramatically altered (Wiesel and Hubel 1963; Wfesel and Hubel 1965). The columns normally activated by the deprived eye shrink, whereas the columns activated by the nondeprived eye persist. Finally, chronic blockade of visual activity with tetrodotoxin impedes the segregation of ocular dominance columns (Meyer 1982; Stryker and Harris 1986).

The overall amount of neural activity during synaptic rearrangements may be less important in synaptic rearrangement than the pattern of neural activity. If both eyes are occluded (presumably retusing activity equally in both ocular pathways), then a substantial shift in ocular dominance columns is not observed (Wiesel and Hubel 1965; see, however, Stryker and Harris 1986). Additional evidence for the importance of the pattern of activity in establishing innervation of the visual system is that profound changes occur in the visual cortex without deprivation if the two eyes are simply made disconjugate by cutting one or more eye muscles, or if the two eyes are occluded alternately during the critical period (Hubel and Wiesel 1965). In both these cases, each eye receives the same amount of stimulation, and at maturity the animals appear to see normally with either eye. Yet recordings of single units from the visual cortex of animals with strabismus or alternating occlusion show a marked reduction in the number of binocularly driven cells. These results led Wiesel and Hubel to suggest that asynchronous activity enhances competition between axons innervating the same cortical neuron, whereas synchronous activity of axons innervating a cell somehow impedes their competitive interaction (Wiesel and Hubel 1965). A similar result has been described in the development of the auditory system (Sanes and Constantine-Paton 1983).

A simple idea that may explain the relationship between trophic factors and activity is that the release and uptake of trophic factors (for which innervating axons evidently compete) occurs most effectively when the pre- and postsynaptic cells are active together (Lichtman and Purves 1981; Purves and Lichtman 1980). In this view, boutons which are simultaneously active on the same postsynaptic cell will get a larger share of positive reinforcement than boutons which are asynchronously active. Such a link would reduce competition between synchronously active terminals and enhance competition between asynchronous inputs. For example, it would promote the innervation of a muscle fiber or ganglion cell lacking dendrites by the terminals of the single presynaptic axon during the period of synaptic rearrangement that normally occurs in developing autonomic ganglia. The protection from competition afforded by synchronous activity might also help explain why several systems (such as the Ia-motor neuron system) show little evidence of focused innervation or synapse elimination. If the afferents were largely activated together, then there would be no way of establishing differences between different sets of terminals, and the positive feedback provided by trophic support would not distinguish different inputs. Tonic muscle fibers (without action potentials) would also not be sites of synapse elimination, because the target cells would never be active when the motor axons were. A similar argument might be made for synapses on dendrites.

SUMMARY

Synaptic rearrangements in early postnatal life are widespread. These rearrangements probably reflect the operation of the few

fundamental rules that govern quantitative apportionment of innervation. It seems likely that axons and target cells in various regions of the nervous system obey the same rules, although this obedience may sometimes be obscured by the anatomy of the particular situation (as in the comparison of muscle fibers and autonomic ganglion cells). A number of influences are evidently at work in this process, including those aspects of target cells that provide for qualitative recognition of appropriate synaptic partners. Competition between axons, however, seems a fundamental theme as well as a driving force in establishing patterns of innervation.

The object of terminal competition may be a trophic factor supplied by the target cell, although the arguments for this view remain circumstantial. The outcome of competition between terminals innervating the same target cell evidently depends on the geometry of postsynaptic cells and the pattern of neural activity among the competitors. The nature of the rearrangements that are observed in most systems that can be carefully studied strongly suggests a link between the positive feedback supplied by the postsynaptic cells and patterns of activity. Some linkage of this sort is required to explain the identification of a set of synapses which arises from a single axon and the unitary capture of individual postsynaptic cells. The immense range of convergent synaptic strength and neural unit configuration in the nervous system is presumably generated by varying a mix of these several influences.

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Drug-Vulnerable Events in Neuronal Development: Implications for the Selection of Test Methodologies

Linda Patia Spear

INTRODUCTION

Exposure to psychoactive drugs early in life may induce long-term behavioral effects resulting from subtle alterations in neural functioning. The developing organism appears to be vulnerable to potential neurobehavioral consequences of drug exposure throughout much of the period of neural development, ranging from the beginning of neural tube closure (occurring on embryonic day 8 (E8) in rats) to the termination of the major brain growth spurt, ending in the rat at about 10 days postnatally (Dobbing and Sands 1979). While gross structural malformations are the frequent hallmark of exposure to chemicals during organogenesis (E8 to 14 in rats), neurobehavioral teratogenic effects may also be observed during this period, even at doses below those required to produce gross teratogenic effects (Hutchings 1978). However, neurobehavioral teratogenic effects are particularly prevalent following drug exposure during the fetal period (El5 to birth in rats) (Hutchings 1978; Buelke-Sam et al. 1985) and the early postnatal period in rats (Diaz and Samson 1980; West et al. 1984; West and Hamre 1985; West et al. 1986), ages refractory to an insult-induced production of gross structural defects.

During the developmental span from neural tube closure to the end of the major brain growth spurt, a multitude of ontogenetic events occur in the nervous system, including much neuronal generation and migration of neuroblasts to assume appropriate positions, the onset of both neurotransmitter synthesis and the formation of receptors for neurotransmitters/neuromodulators, the initial elaboration of axonal and dendritic processes, and the beginnings of gliogenesis (Jacobson 1978). The exact sequencing and scheduling of these events vary markedly with the neuronal population under consideration, with critical variables including basal or alar plate derivation, final location of neuronal group or terminal projections, size of neurons within population, neurotransmitter substance utilized, etc. (Rodier 1980; Lauder and Krebs 1984). Because of the diverse complexity with which these events unfold, an insult sustained at any particular point in ontogeny may have

the potential to disrupt a number of these processes concurrently in different neural regions throughout the nervous system. Although cells that are actively proliferating appear to be particularly vulnerable to disruption by chemical insults at any particular developmental stage (Hutchings 1978; Rodier 1980), other processes of neuronal development and differentiation also appear to be susceptible to drug-induced alterations early in ontogeny.

There are a number of different ways in which a chemical insult can influence neurobehavioral development. These mechanisms can be divided into three categories: (a) "nonspecific" mechanisms; (b) general influences on neuronal generation and differentiation; and (c) specific influences on the development of the neurotransmitter system(s) upon which the drug exerts its central effects (as well as closely related neural systems) (Lauder and Krebs 1984; Boer and Swaab 1985).

NONSPECIFIC MECHANISMS BY WHICH CHEMICAL INSULTS MAY INDUCE NEUROBEHAVIORAL TERATOGENIC EFFECTS

"Nonspecific" mechanisms include those that influence neurobehavioral development through indirect effects of the drug, as opposed to direct action of the drug on the developing nervous system. Exposure to the test substance may induce hormonal and physiological reactions in the treated dams, which could lead to alterations in offspring neurobehavioral development; even relatively mild stress of dams during pregnancy has been shown to alter subsequent behavior, hormonal responsiveness, and neurochemical function in offspring (Ader and Plaut 1968; Meisel et al. 1979; Peters 1982). Drug-induced hormonal reactions, including but not restricted to those associated with stress reactions, may also serve to alter subsequent neurobehavioral development in offspring. For instance, ethanol-induced increases in maternal corticosterone levels have been suggested to be a major contributing factor to the behavioral, neurochemical, and physiological alterations observed in offspring thereby exposed to the hormone through the placenta or milk (Angelucci et al. 1985). Given that the developing organism itself is capable of secreting corticosterone from the later days of gestation, it is possible that chronic drug treatment in late gestation or postnatally may also directly stimulate corticosterone release in the offspring, which also could contribute to the observed pattern of neurobehavioral alterations induced by early drug exposure. Many drugs induce alterations in body temperature, including hyperthermia. Such hyperthermia could also contribute to the pattern of neurobehavioral alterations induced by a test substance, given that hyperthermia per se is known to induce teratogenic and behavioral teratogenic effects (Edwards 1968). Drugs that induce vasoconstriction, such as cocaine, may result in constriction of the umbilical artery, resulting in some degree of fetal hypoxia (Mahalik et al. 1984). which is known to induce behavioral alterations in offspring (Mactutus and Fechter 1986).

There are a number of ways in which drug exposure may alter the nutritional status of the offspring, which could contribute to the pattern of observed effects, given that early life undernutrition has been observed to induce marked neurobehavioral alterations in offspring (e.g., Morgane et al. 1978). Drug-induced alterations in placental blood flow may alter not only the passage of oxygen to the fetus, as discussed above, but also the passage of other nutrients to and wastes from the fetus, with subsequent long-term effects (Mahalik et al. 1984). Certain psychoactive drugs may also lead to a reduction in food intake by gravid dams, and hence malnourishment in the pups. For this reason, in cases where a drug is expected to reduce ingestion, experimenters often use a pair-feeding regimen in which some control animals are given restricted feedings based on the consumption of diet by drug-treated animals. It should be realized, however, that although such pair-feeding designs control for nutritional factors to some extent, this design does not permit detection of potential interactions of the drug treatment with nutritional deprivation. A final way in which drug exposure may alter the nutritional status of the offspring is via alterations in efficacy of the suckling process, either by influencing the nutritional content of milk or the proclivity of the dams or pups to engage in the process of suckling. As discussed below, these possibilities can be largely, but not completely, eliminated by fostering gestationally exposed offspring to surrogate dams at birth.

Early exposure to drugs may influence neurobehavioral development of offspring indirectly by altering maternal/offspring interactions, with subsequent long-term effects on offspring behavior and brain function. This is a particular problem when the drug treatment is given postnatally, either to the dams or the offspring, where even subtle alterations in the behavior of pups or the dam may lead to marked disruptions in maternal behavior. For this reason, some investigators using postnatal administration regimens have resorted to rearing pups artificially (Diaz and Samson 1980; West et al. 1984; West et al. 1986; West and Hamre 1985). In a prenatal treatment regimen, pups can be fostered to surrogate dams shortly after birth to eliminate potential treatment effects upon maternal behavior or milk production. However, behavioral differences between drug-treated and control pups may still influence the amount of maternal attention given to the pups even with fostering. The two different procedures for fostering currently in use are cross-fostering (mixing drug-treated and control animals within a single litter) and surrogate fostering (placing an entire litter of pups with a foster dam). Given that subtle behavioral differences between prenatally drug-treated and control pups (leading to differential maternal attention) may be relatively magnified in a cross-fostering design because of the "contrast" effect of having both normal and drug-treated pups in the same litter, surrogate fostering has been suggested to be the preferred method for rearing prenatally treated pups (Hutchings 1985).

A final indirect way in which early drug exposure may influence long-term neurobehavioral functioning is through altering the interaction of brain and behavior during development (Swaab and Mirmiran 1985). The primary neural effects of a teratogen may lead not only to secondary disruptions in the development of other neural substrates but also to alterations in the ability of the young organism to emit behaviors and interact and adapt appropriately *to* its immediate environment. These indirect neural and/or behavioral effects of the neurobehavioral teratogen may further alter the prospectus for future development, contributing to the pattern of alterations in neurobehavioral functioning observed as the animal matures. Only a few examples will be given to illustrate the diversity of this type of indirect effect. Early drug insults may interfere with the development of critical fetal or infant behavior patterns necessary for normal subsequent development. For example, even low levels of alcohol administered acutely have been observed to markedly depress motor movements in fetal rats (Smotherman et al. 1986) and breathing in human fetuses (Fox et al. 1978; McLeod et al. 1983); such fetal movements (Burns 1986) have been suggested to be critical for normal maturation (Hofer 1981; Smotherman et al. 1986). Postnatally, deficits in suckling behavior in neonates potentially induced, for example, by early insult-induced depressions in serotonergic activity (Adrien 1978; Isaacson et al. 1977; Spear et al. 1985; Riley et al. 1985) may affect the nutritional status of the offspring, which may lead to additional neurobehavioral complications only indirectly related to the drug treatment per se. Drug-induced electrophysiological alterations may also influence neurobehavioral maturation. For instance, a number of drugs (e.g., clonidine, clomipramine) have been observed to suppress "active" (REM) sleep during development; such drugs induce marked behavioral alterations later in life, along with disturbed sleep patterns and reductions in cortical size, perhaps in part related to the drug-induced suppression of this predominant state of sleep in young organisms (Swaab and Mirmiran 1985).

DRUG-INDUCED ALTERATIONS IN CENTRAL NERVOUS SYSTEM (CNS) GENERATION AND DIFFERENTIATION

Through such diverse mechanisms as those discussed above, drugs administered early in life may influence neurobehavioral functioning of exposed offspring. Drugs may also directly influence brain development through altering functional levels of neurotransmitter substances suggested to play an important role in the modulation of neural tube closure, cell generation, migration, and differentiation, beginning even prior to the developmental onset of neurotransmission (Lanier et al. 1976; Tomkins 1975; Lauder and Krebs 1984). For instance, drugs that depress monoamine levels have been reported to disrupt neural tube closure and early stages of neurulation long before nascent adult-typical monoaminergic-containing neurons are formed (Lawrence and Burden 1973; Jurand 1980; Palen et al. 1979). Later in ontogeny, monoaminergic neurons, among the earliest forming neural populations in brain (generated between E12 to 14 in rats), have been suggested to

modulate the genesis and differentiation of later developing neuronal systems (Lauder and Krebs 1984). For example, pharmacological treatments that lower serotonin levels in fetuses have been reported to delay or reduce neurogenesis of neurons destined for a variety of later forming brain regions (Lauder and Krebs 1984). Postnatal cell replication in brain has also been observed to be reduced following pharmacologically induced increases or decreases in catecholamine levels in infant rats (Barochovsky and Patel 1982). Endorphins (Zagon and McLaughlin 1983) and other neuropeptides, such as adrenocorticotrophic hormone, thyrotrophic-releasing hormone, and somatostatin (Boer and Swaab 1985; Handelmann 1985), have also been suggested to play a role in brain growth and neuronal proliferation.

Exposure to drugs early in life may also directly alter functioning of specific neurotransmitter systems that may serve to regulate neuronal differentiation processes per se. For instance, histamine and norepinephrine have been suggested to be trophic factors during a critical postnatal period because of their role in modulating the activity of ornithine decarboxylase, an enzyme involved in the synthesis of polyamines which play key roles in the regulation of cellular development and differentiation (Morris et al. 1983). Norepinephrine has also been suggested to play a critical role in maintaining neural plasticity in the visual cortex during a critical postnatal period, with reports that drug-induced disruptions in norepinephrine levels lead to a loss of normal developmental plasticity in this brain region (Kasamatsu et al. 1984; Kasamatsu 1985).

DRUG-INDUCED ALTERATIONS IN THE DEVELOPMENT OF NEUROTRANSMITTER SYSTEMS

Chronic drug administration may also directly influence development of the specific neurotransmitter system(s) upon which the drug exerts its central effects and closely related neural systems via transsynaptic mechanisms (Lauder and Krebs 1984; Boer and Swaab 1985; Kellogg 1985). Although the available data are somewhat limited, it appears that in many, but not all, instances the neural systems altered by administration of drugs during gestation are often some of the same neural systems affected by acute or chronic administration of these drugs in adult animals. The nature of these changes in the affected neural systems after chronic gestational administration, however, may be in the opposite direction from, and relatively permanent when compared to, compensatory responses induced by chronic exposure to the same drug during adult life.

For instance, treatment with dopamine antagonists early in life has been observed to result in alterations within the dopaminergic system and related neural systems that are generally in an opposite direction from those compensatory responses seen following chronic treatment with these drugs later in life (Spear and Scalzo 1986). After withdrawal from chronic neuroleptic treatment in adulthood, animals typically exhibit an enhanced response to

dopamine agonists (e.g., amphetamine and apomorphine) (Muller and Seeman 1978; Spear et al. 1980) and attenuated responses to dopamine antagonists (e.g., haloperidol) (Asper et al. 1973; Thornburg and Moore 1975) and cholinergic agonists (e.g., arecoline) (Dunstan and Jackson 1977; Gianutsos and La1 1977). These psychopharmacological alterations presumably result, in part, from the production of dopamine postsynaptic receptor supersensitivity, which has been characterized (using ^{3}H-haloperidol as a ligand) by an increase in receptor number with no change in affinity (Burt et al. 1977). Oopamine presynaptic autoreceptors in striatal and mesolimbic dopamine regions also appear to be supersensitive following adult chronic treatment with neuroleptics (Roth 1979).

In contrast, chronic early haloperidol treatment has been reported to produce an attenuated response to amphetamine (Spear et al. 1980) and apomorphine (Rosengarten and Friedhoff 1979) and accentuated responses to haloperidol and arecoline (Spear et al. 1980; Shalaby and Spear 1980)--psychopharmacological response profiles opposite to those seen following chronic neuroleptic treatment in adulthood. Following early chronic exposure to either direct (haloperidol) or indirect (alpha-methyl-para-tyrosine) dopamine antagon'sts (Rosengarten and Friedhoff 1979; Rosengarten et al. 1983), ^{3}H-spiroperidol binding is decreased--again, an opposite response from that seen after chronic adult neuroleptic exposure. Similarly, whereas dopamine autoreceptors have been reported to be supersensitive following chronic neuroleptic treatment in adulthood, they have been reported, conversely, to be hyposensitive after chronic neuroleptic treatment throughout gestation and the preweaning period (Shalaby and Spear 1980; Scalzo and Spear 1985). Thus, neural modifications induced by chronic neuroleptic treatment during development often appear to be opposite those induced by such treatment in adulthood.

As shown in table 1, it appears that treatment during the prenatal period is critical for producing these effects. For instance, treatment with haloperidol during the prenatal period alone decreases ^{3}H-spiroperidol binding and apomorphine-induced stereotyped behavior (Rosengarten and Friedhoff 1979), with the critical period for these effects apparently occurring during gestational days 15 to 18 (Rosengarten et al. 1983). Early postnatal treatment, conversely, has been observed to produce an adult-typical profile of increased receptor binding and apomorphine-induced stereotyped behavior. Thus, it appears that, whereas postnatal neuroleptic treatment alone produces compensatory responses within the dopaminergic system that are often similar to those seen after treatment in adulthood, prenatal treatment (or combined pre- and postnatal treatment) at the time when these receptors are first developing produces long-term effects opposite to those occurring in response to chronic neuroleptic treatment in adulthood.

The outcome of chronic neuroleptic treatment early in life *vs.* in adulthood also appears to differ in longevity. The neurochemical and psychopharmacological alterations induced by chronic neuroleptic treatment in adulthood dissipate rapidly, within days or

TABLE 1. *Comparison of the psychopharmacological and neurochemical alterations produced by chronic haloperidol treatment during development or in adutthood*

	Following Early Chronic Treatment (Tested In Adulthood)			Following Adult Chronic Treatment
	Prenatal Treatment	Combined Pre- and Postnatal Treatment	Postnatal Treatment	
Amphetamine responsiveness	--	Attenuated	--	Accentuated
Haloperidol responsiveness	--	Accentuated	--	Attenuated
Arecoline responsiveness	--	Accentuated	--	Attenuated
Dopamine autoreceptor sensitivity	--	Hyposensitive	No difference	Supersensitive
Apomorphlne-induced stereotypy	Attenuated	No difference	Accentuated	Accentuated
Dopamine receptor binding	Decreased	--	Increased	Increased

NOTE: "--" means that this combination of treatment and test has not been assessed.

several weeks after termination of the drug treatment regimen (Muller and Seeman 1978). In contrast, the effects of chronic neuroleptic treatment early in life reviewed above are long-lasting, appearing to be at least as evident when examining animals in adulthood rather than shortly after treatment termination. These alterations may persist into older adulthood as well, given that dopamine autoreceptor hyposensitivity induced by chronic haloperidol treatment early in life is seen not only in young adults (2 to 3 months old) but also in older adult rats (12 to 13 months old) (Scalzo and Spear 1985).

There are a number of interesting aspects about the receptor alterations induced by early drug treatments. As the affinity of receptors remains constant during normal development (Balazs and Brooksbank 1981; Bruinink et al. 1983), so the affinity remains constant following the early drug treatment, with the decrease in DA receptor binding (following prenatal neuroleptic treatment) being associated with a decrease in the number of binding sites with no change in their affinity (Rosengarten and Friedhoff 1979). Alterations in dopamine receptor binding can also be seen in the opposite direction following early drug treatment, with chronic exposure to the dopamine precursor L-DOPA during the end of the second and the beginning of the third trimester inducing an increase in DA receptor binding (Friedhoff and Miller 1983). Thus,

treatment with either a dopamine agonist or antagonist at the time when dopamine receptors are first developing seems to have a long-term effect on the number of dopamine receptors present in adulthood, even though only a small proportion of the total number of dopamine receptors eventually present in the adult are evident during the prenatal period of drug exposure (Pardo et al. 1977; Deskin et al. 1981). Given that in a number of neuronal systems, including the dopaminergic system, receptors appear to develop prior to afferent input to these receptors (Woodward et al. 1971; Coyle and Campochiaro 1976; Coyle et al. 1984), it appears that these drug-induced alterations in the "setting" of the number of receptors eventually to be formed may occur at least partially prior to the development of structured synaptic connections.

The lack of data makes it impossible to determine at present whether it is only in the dopaminergic system that manipulating neurotransmitter availability for associated receptors in their nascent stage results in alterations in the setting of the population number of those receptors. Indeed, evidence suggests that this may not occur with the benzodiazepine receptor system at least. Kellogg (1985) has reported that prenatal exposure to diazepam does not produce any marked alterations in the characteristics of the benzodiatepine receptor binding site but alters other neural systems. It is interesting to note, however, that opposite responses following drug treatment during development and in adulthood have been observed not only at the receptor level but also behaviorally. As Kellogg expresses it:

> In some instances, the effect on the offspring from prenatal exposure to certain drugs has been similar to the effects of maternal conditions for which the drugs might be prescribed. (Kellogg 1985, p. 150)

In other words, developmental drug exposure may induce long-term behavioral alterations-analogous to the behaviors for which the drug is useful in treatment in adulthood.

IMPLICATIONS FOR THE SELECTION OF TEST METHODOLOGIES IN NEUROBEHAVIORAL TERATOLOGICAL RESEARCH

As discussed above, there are a substantial number of ways in which any given psychoactive substance could influence neurobehavioral functioning. Recognizing these potential mechanisms what implications can be derived for the selection of appropriate test methodologies in behavioral teratological and neuroteratological research? In all instances examined, once the nervous system has matured to a point where it is capable of responding to acute exposure to a given drug, it appears that the drug acts directly on the same neural systems via the same mechanisms of action that it has in adulthood. Thus, to predict the potential neurobehavioral teratogenic effects of a drug whose central effects are known, one should consider the adult-typical mechanisms of action of the drug and what roles those neural systems may play in the developmental process. For instance, when assessing the

neurobehavioral teratogenic effects of drugs that influence levels of neurotransmitters/neuromodulators involved in regulating the process of neuronal generation and differentiation, it would appear critical to examine neural measures sensitive to the action of these regulatory or trophic substances and behavioral measures previously linked to disruption of neural generation at the ages when the treatment is administered (Rodier 1980).

Given that drug administration early in life often influences the development of the neurotransmitter system(s) upon which the drug exerts its central effects, it would also appear to be particularly useful to focus examination on neurochemical measures of that neurotransmitter system and on behavioral measures previously shown to be sensitive to acute alterations in those neural systems (Kellogg 1985; Geyer and Reiter 1985). Indeed, it has been argued that a generalized behavioral test screen for use with any potential teratogenic compound may be of questionable appropriateness when working with substances whose central effects in adult animals have been well characterized (Kellogg 1985).

It is much more difficult to predict the neurobehavioral consequences of drugs whose mechanisms of action are unknown. In the absence of any prior data on the potential behaviors and neural systems that may be affected by the substance, it has been suggested that initial testing should include general "apical" behavioral tests which are rather global in nature and likely to reflect alterations in any of a number of neural systems (e.g., locomotor activity and learning/performance tasks) (Geyer and Reiter 1985). A recent workshop to discuss strategies for the selection of test methods in behavioral teratology has identified a number of categories of functional effects that should be examined in initial apical tests: (1) physical growth and maturation; (2) reflexes; (3) motor development/activity; (4) sensory/attentional; (5) affective; (6)cognitive (learning/memory/performance); and (7) reproductive behavior (Geyer and Reiter 1985). Neural measures to be assessed would also have to be very broad-based initially, or perhaps delayed until after sufficient behavioral testing is conducted to provide suggestive evidence regarding what potential neural systems might be affected. Although the broad-based nature of the behavioral teratogenic tests used has occasionally been criticized by those outside the field as being atheoretical and essentially "fishing expeditions," in the absence of any prior data on the potential behaviors and neural systems that may be affected, this broad-based approach is considered to be essential in initial work, to be followed ideally by more specific tests as needed to characterize specific functional alterations (Geyer and Reiter 1985). Even when prior knowledge of the mechanism of action of the test substance can provide some guidance for the choice of appropriate test methodologies, numerous potential nonspecific effects of the drug treatment make it possible, if not likely, that unpredicted consequences may also occur--consequences that may be undetected if only very specific tests are used. Apical tests may also provide useful adjunct tests for such nonspecific effects in conjunction

with more specific test methodologies directed toward examining alterations induced by the known mechanisms of action of the test substance.

There is one last important variable to be considered when selecting test procedures to be used in neurobehavioral teratology experimentation: the consideration of testing age (Spear 1984). Although most testing of neurobehavioral teratogenic effects has been conducted in adult offspring, testing throughout the lifespan would appear to be essential to determine the true neurobehavioral teratogenic potential of a substance. For instance, alterations induced by early drug exposure, although effectively masked in adulthood, may become more evident with the decline in neural function seen during the aging process, perhaps leading to premature senescence or death (Spyker 1975). As another possibility, drug-induced neurobehavioral alterations could be considered to be a special case of early brain damage, with perhaps substantial recovery occurring in some cases following insults that occur during gestation (Riley et al. 1985). A focus on testing in infancy is not only possible, in view of recent advances in developmental psychobiology, but also perhaps critical. Given that the limited data available on neurobehavioral teratogenic consequences in humans are typically derived shortly after birth, correlations between human and animal data might be expected to be closer when analogously assessing the behavior of the test animal population early in life. Examining the potential teratogenic effects of test compounds in developing animals may additionally provide more direct indices regarding the underlying primary direct alterations induced by teratogens than testing animals in adulthood. As discussed previously, the direct neural effects of a teratogen may lead not only to secondary disruptions in the development of other neural substrates but also to alterations in the ability of the young organism to interact and adapt appropriately to its immediate environment. Each of these teratogen-induced effects, alone and in combination, may alter the prospectus for future development, gradually leading to a concatenation of alterations in neurobehavioral functioning as the animal matures that may make it progressively difficult to determine the underlying primary alterations induced by the neurobehavioral teratogen (Spear et al. 1985). Research using a developmental approach to examine systematically the neurobehavioral teratogenic effects of a variety of drugs of use and abuse, using testing methodologies specifically chosen on the basis of the known neural actions of the test compound, has the potential to reveal a great deal about the processes of normal neural maturation and how they are altered by chemical insults early in life.

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Alcohol and Brain Development

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INTRODUCTION

There is a story (which may or may not be true) that Alice B. Toklas, the long-time confidante of Gertrude Stein, leaned over her dying friend and whispered, "Gertrude, what is the answer? What is the answer?" After a moment, Gertrude wrinkled her brow, raised her head slightly, and replied, "What was the question?"

There is considerable relevance in that vignette to the problems in identifying teratogenic alterations in the central nervous system (CNS) associated with the abuse of substances such as alcohol. Experimental strategies for research directed toward a suspected teratogen depend, in large part, on the types of questions which can be addressed, which in turn depend on the level of progress in that area of research at that particular time. Formulating the appropriate questions is fundamental to progress in understanding neuroteratological effects of substances of abuse in general and of alcohol in particular. In this paper, we illustrate a few of these issues by presenting some of the data from our laboratory on changes in brain development resulting from perinatal alcohol exposure. In doing so, we trace the evolution of our questions and experimental approaches to fetal alcohol effects using animal model systems (while taking some liberties with the chronological order of these questions). As a consequence, this paper takes a somewhat unusual approach in that we focus as much on the choice of questions as on the data.

One of the most basic questions of neuroteratology, and one that is generally the first asked, is whether a particular substance can adversely affect the structural and functional development of the brain. In the case of ethyl alcohol, the possible teratogenic effects were suspected for centuries (Rosett 1976), but the fetal alcohol syndrome (FAS), expressed as a characteristic pattern of craniofacial abnormalities and varying degrees of CNS dysfunction, was not formally defined in humans until 1973 (Jones-and Smith 1973; Jones et al. 1973). The status of alcohol as a teratogen was obscured because many women who abused alcohol also abused

other drugs and suffered from nutritional deficits. Thus, it was not always possible to demonstrate whether the observed birth defects were caused by the alcohol or were a function of other factors. Since it is unethical to give alcohol to pregnant women in controlled studies, the task of answering most of the questions pertaining to possible alcohol-induced birth defects fell to animal research.

GOALS OF ANIMAL MODEL RESEARCH

The most serious fetal alcohol effects in surviving FAS infants are varying degrees of mental retardation (Clarren 1986; Streissguth 1986). Therefore, a significant amount of fetal alcohol research in animals has focused on the CNS (West 1986). An initial goal of this research was to document whether alcohol could disrupt normal development. A number of well-controlled animal studies over the last decade have demonstrated a variety of neuromorphological (Colangelo and Jones 1982; Davies and Smith 1981; Hammer 1986; Phillips 1986; Pettigrew 1986; Samson 1986; West and Pierce 1986), neurochemical (Druse 1986), hormonal (Weinberg et al. 1986). and behavioral (Bond 1986; Riley et al. 1986) alterations associated with perinatal alcohol exposure.

For most substances, it is sufficient to demonstrate that they have teratogenic properties. However, for substances of abuse, such a demonstration is just the first goal of experimental research, since, as in the case of alcohol, simply publicizing that it is teratogenic is not enough to eliminate the health hazard. Even the Surgeon General's (1981) recent advice to abstain from alcohol during pregnancy has failed to prevent some pregnant women from drinking heavily. Subsequently, the major goals of fetal alcohol research have changed. Questions now should be formulated that focus on more sophisticated problems, such as the mechanisms that underlie the fetal damage and the factors that contribute to the severity of such damage. Eventually, these and other approaches should lead us to address the prospects for the prevention of FAS or therapy for offspring of mothers who drank during pregnancy.

Now that alcohol has been shown to act as a teratogen and to disrupt brain development, it has become important to consider more specific questions concerning factors that affect the relative risk of fetal alcohol effects. One of the questions most frequently asked is, How much alcohol is harmful? This seemingly simple question is actually extremely complicated and may be unanswerable in such a simple form. In terms of the CNS, it is now clear that the minimum amount of alcohol that is harmful to development depends on several factors, including the blood-alcohol levels achieved, the pattern of consumption (or exposure), the period of development in which alcohol exposure occurs, and the particular brain regions and types of cells under consideration.

SELECTING AN ANIMAL MODEL

In order to evaluate the large number of animals necessary for dose-response studies of the effects of alcohol, it was necessary to find a CNS fetal alcohol effect that was easy to measure yet was sensitive to the teratogenic effects on the brain, and at the same time, was relevant to FAS in humans. Brain growth, as indicated by brain weight/body weight ratios, is such a measure. Since microcephaly (a smaller than normal head) is a primary feature of FAS (Jones and Smith 1973; Streissguth et al. 1980), microencephaly (a smaller than normal brain for body size) was considered an appropriate measure to incorporate into our animal model research. Samson and his associates (Diaz and Samson 1980; Samson and Diaz 1982; Samson and Grant 1983) demonstrated the effectiveness of using brain weight and the brain weight/body weight ratio as a measure of alcohol's effects on brain development during the early postnatal brain growth spurt in the rat, a period of brain development roughly equivalent to the human third trimester. Samson (1986) showed that, in the rat, microencephaly resulted from alcohol exposure during the third trimester equivalent in the absence of decreases in body weight, but prenatal exposure (encompassing the first and second trimester equivalents) produced decreases in body weight as well as brain weight. In the third trimester equivalent model, microencephaly is an excellent measure upon which to focus these dose-response studies.

In order to model the effects of exposure during the third trimester, it is necessary to expose the rat to the suspected teratogen during the first 2 weeks after birth. Not surprisingly, there are several problems related to nutrition and experimental control associated with administering a drug such as alcohol to neonatal rats (Samson and Diaz 1982; Abel and Riley 1986). In order to control some of these problems, we used an artificial rearing technique (West et al. 1984b; West et al. 1984c). Although it isolates a pup from its mother and littermates, it allows precise manipulation of the dose and rate of delivery of alcohol while maintaining adequate nutritional intake and body growth. Also, in addition to using control groups reared artificially without exposure to alcohol, we standardly include normally reared rats to evaluate the effects of the rearing procedure per se.

PATTERN OF ALCOHOL EXPOSURE

Using brain weight/body weight ratios as a measure of microencephaly, we began our investigations of factors that contribute to the severity of CNS fetal alcohol effects. One of the most obvious factors is the amount of alcohol exposure. We were now back to our original question: How much alcohol is harmful? Our first dose-response analysis of alcohol exposure during part of the early postnatal brain growth spurt (postnatal days 4 to 10) used relatively continuous exposure to alcohol in four doses ranging from 6.6 g/kg/day to 9.7 g/kg/day, with each dose divided into 12 fractions each day and delivered every 2 hours. Not surprisingly, the extent of microencephaly was a function of dose, with

the higher doses producing the greatest restriction of brain weight/body weight (Pierce and West 1986a), corroborating the work of others (Samson and Grant 1983). More importantly, there was substantial variability at each dose, both in the brain weight/body weight ratios at postnatal day 10 and in the blood-alcohol concentrations (BACs) measured on day 6. The brain weight/body weight ratios were more highly correlated with BAC than with dose (Pierce and West 1986a).

Since BAC appeared to be important in the appearance of microencephaly, we predicted that anything that operated to increase the peak BAC would increase the amount of microencephaly. It is impossible to dissociate BAC from dose, since BAC is a function of the amount of alcohol given plus the rate of delivery. Webster and his associates (1983) provided an interesting approach to this problem. They found that two injections of alcohol (2.9 g/kg) spaced 4 hours apart induced more malformations than if the injections were spaced 6 hours apart. The fact that the 4-hour interinjection interval produced higher peak BACs than injections spaced 6 hours apart suggested that the peak BAC, rather than the total amount of alcohol injected, was a crucial factor in those teratogenic effects of alcohol. We hypothesized that manipulation of the peak BAC would also be an important determinant of the severity of alcohol-induced alterations in the CNS during later periods of development.

These considerations suggest that the alcohol consumption pattern of the pregnant woman, or, in animal studies, the method of exposure of the animal to alcohol, can determine the peak BAC and thereby the risk of fetal alcohol effects. Most animal models of fetal alcohol exposure use forced consumption of alcohol via a liquid diet by the dam during gestation, and these studies have not been directly concerned with the pattern of alcohol consumption and the resulting pattern of BACs. Since the dam primarily controls the intake of alcohol in these models, experimental manipulation of the pattern of alcohol exposure is difficult to achieve. Artificial rearing allows careful and precise control over the concentration and rate of delivery of the alcohol. Thus, exposure of the pup to alcohol via artificial rearing during postnatal days 4 to 10 provides an excellent method of testing the effects of different patterns of exposure while maintaining a constant daily amount of alcohol.

In testing the effect of manipulating the daily pattern of exposure to alcohol, we used a dose of 6.6 g/kg/day, which did not induce significant brain growth deficits when given uniformly in fractions delivered every 2 hours from postnatal days 4 to 10 (Pierce and West 1986a). We reasoned that, if a given amount of alcohol that was just below threshold for inducing microencephaly (when given in a uniform daily pattern that did not produce high BACs) were condensed into a shorter period each day (so that the peak BACs were higher), this condensed treatment would restrict brain growth if the peak BAC were, in fact, a key factor in producing microencephaly. The uniform delivery of the 6.6 g/kg/day

dose produced BACs that were low (80 mg/dl) and stable over 24 hours. When the same daily amount was condensed into just 12 hours of each day, the resulting BACs were cyclic, with peaks near 270 mg/dl. This condensed treatment significantly limited brain growth (Pierce and West 1986b). Furthermore, when the same dose was condensed even more (into just 8 hours of each day), the peak BAC was even higher (near 400 mg/dl), and brain growth was even more severely restricted (West et al., in press) (figure 1). In further support of the hypothesis that peak BAC is a key factor in the amount of alcohol necessary to produce microencephaly, we found that the condensed alcohol exposure, but not the uniform alcohol exposure, produced permanent effects on brain growth and behavior (Kelly et al., in press). Taken together, these findings strongly suggest that any condition that tends to produce high BACs increases the risk that any dose of alcohol will affect brain growth and behavior. In the human situation, women alcoholics tend to binge rather than drink continuously (Madden and Jones 1972), and FAS children have been born to binge drinkers (Clarren et al. 1978; Hermann et al. 1980). While women who drink during pregnancy usually decrease the amount of alcohol they consume during the third trimester, when they do drink during this period, they often binge drink (Stephens 1985)--a situation perhaps similar to the condensed dose version of our third trimester model.

TEMPORAL VULNERABILITY

The second variable that we investigated that might affect the threshold for CNS damage was temporal vulnerability. In this case, the question was whether there is a particular period when alcohol is especially harmful to the development of the brain. If so, this would make the threshold for CNS damage variable across time and would suggest that the state of brain development is an important factor in the sensitivity of the brain to teratogenic effects of alcohol.

The study of possible critical periods of a teratogen's effects on the CNS is complicated because of the complexity of the brain itself and because it develops over an extremely long period. In addition, although the brains of all mammals exhibit the same general stages of development, the timing of those stages, relative to birth, can be quite different (Dobbing and Sands 1979). The period of most rapid brain growth, often called the brain growth spurt, occurs during the third trimester and continues after birth in humans (Dobbing and Sands 1973). In species (such as the rat) that are less developed at birth than humans, the brain growth spurt occurs after birth during the first 2 postnatal weeks. In other words, in terms of brain development, the entire gestation in the rat is equivalent to only the first and second trimesters in humans. This difference in developmental timing is significant if rats are used as a model system for studying CNS defects with the intention of extrapolating the results to humans.

Another problem that complicates the timing issue is that, even within a particular species, brain development is heterogeneous in

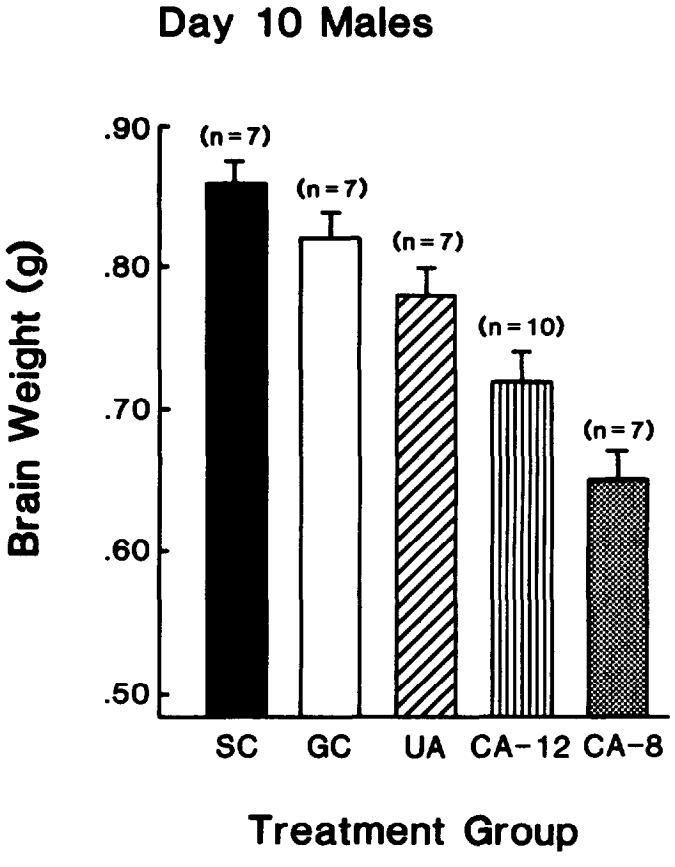

FIGURE 1. *Variations in the extent of restricted brain growth (microoencephaly) in 10-day-old male rats induced by the same daily amount of alcohol administered in different patterns during postnatal days 4 to 10 (third trimester equivalent)*

NOTE: The three groups of rats given alcohol received 6.6 g/kg/day. The uniform alcohol (UA) exposure group received the alcohol distributed uniformly (in 12 fractions 2 hours apart) during each 24-hour period. The condensed alcohol (CA-12 and CA-8) exposure groups received the alcohol condensed into 12 and 6 hours, respectively, In each 24-hour period. The gastrostomy control (GC) group was treated In the same manner as the UA group, except that an isocaloric amount of maltose-dextrin was substituted for the alcohol. The suckle control (SC) group was reared normally by dams. Note that as the dally period of exposure became shorter, resulting in progressively higher BACs, the amount of microencephaly became greater.

that not all brain regions develop at the same time or at the same rate. While the neurons in most nuclei are generated during the second trimester equivalent, there is considerable variation in the onset and completion of neurogenesis across different regions. In addition, a few regions have populations of both large and small cells that develop over extended and different periods. For example, the hippocampus, cerebellum, and olfactory bulb each have populations of large cells that are generated during the second trimester equivalent, and populations of small cells that are formed primarily during the third trimester equivalent.

The Hippocampus as a Model System

We examined several aspects of these interrelated questions of differential vulnerability to alcohol due to differences in the developmental timing of the alcohol exposure and regional or cellular differences in the brain by focusing on the hippochmpal formation. This strategy provided several advantages. The cellular and synaptic organization of the hippocampus and dentate gyrus is relatively simple, and the source and pattern of termination of intrinsic and extrinsic connections are reasonably well known (Isaacson and Pribram 1986). The development of the cells of the hippocampal formation has also been well studied (Schlessinger et al. 1975; Schlessinger et al. 1978; Bayer 1980), and, of the two primary cellular components, the hippocampal pyramidal cells are generated prenatally in the rat, while the dentate granule cells are generated almost exclusively after birth. This fortuitous circumstance allowed us, using the third trimester equivalent model, to determine the effect of alcohol exposure on one population of neurons that was postmitotic and another that was undergoing proliferation. Finally, the hippocampal formation has been used extensively in rats to study neuroplasticity, particularly the reactive synaptogenesis induced in mature rats by lesions of major sources of afferents such as the entorhinal cortex. Thus, the hippocampal formation provides a good system for examining several aspects of teratogenic effects of early alcohol exposure on neuronal development and plasticity in animal model research.

Effects of Alcohol on Developent of Mossy Fibers

Our earliest specific neuromorphological studies concerned the effects of alcohol on the development of the mossy fiber projection from the granule cells of the dentate gyrus to the pyramidal cells in field CA3 of the hippocampus. Our first questions were: Does prenatal alcohol exposure affect the normal development of this CNS projection? Are there regions of the brain or different cell populations that are especially vulnerable to alcohol, i.e., have a lower threshold for damage, during development? In our earliest study, we administered a liquid diet containing either 35-percent ethanol-derived calories or an isocaloric amount of maltose-dextrin to pregnant rats from day 1 to day 21 of gestation, i.e., the period of brain development equivalent to the first and second trimesters in humans. At birth, all pups were fostered to surrogate mothers and weaned at 21 days. After the

rats reached adulthood, brain sections were processed with the Timm stain, and the hippocampi were examined for alterations in laminar staining. As shown in figure 2, the mossy fiber system--the major output from the dentate gyrus to the hippocampus proper--contained an aberrant distal infrapyramidal terminal field

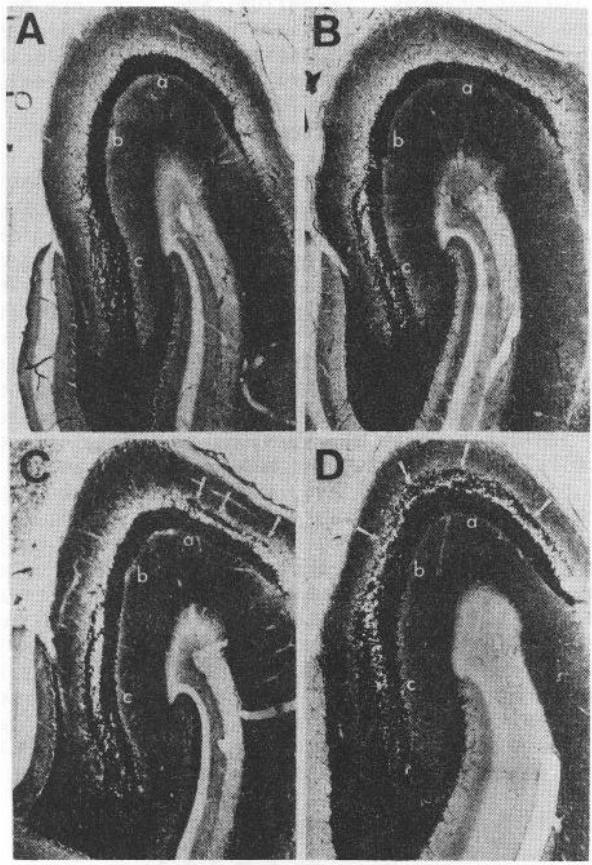

FIGURE 2. *Timm-stained horizontal sections through the mid-temporal hippocampi of adult rats treated previously with alcohol during different periods of development*

KEY: (A) Treatment during gestational days 1 to 10 (first trimester equivalent). (B) Treatment during gestational days 11 to 21 (second trimester equivalent). (C) Treatment during gestational days 1 to 21 (first and second trimester equivalents). (D) Treatment during postnatal days 1 to 10 (third trimester equivalent).

NOTE: The letters a, b, and c denote subfields of hippocampal field CA3. Arrows indicate the aberrant infrapyramidal mossy fibers. The control groups (not treated with alcohol) were similar to the groups given alcohol In gestational days 1 to 10 (panel A) and gestational days 11 to 21 (panel B) and are not presented In this figure.

at midtemporal levels in the rats given alcohol throughout gestation (West et al. 1981; West and Hodges-Savola 1983). Other areas of the hippocampal formation, especially the dentate gyrus, appeared normal in the Timm-stained material. The presence of these alcohol-induced aberrant mossy fibers was confirmed using the anterograde transport of horseradish peroxidase (HRP) (West and Pierce 1984). We also used the anterograde transport of HRP to investigate whether the terminal fields of the major afferents to the dentate gyrus were altered by the alcohol exposure. As with the Timm-stained analysis, the position and size of the commissural (Dewey and West 1985b) and perforant pathway (Dewey and West 1985c) projections to the dentate molecular layer *were* unchanged. Thus, the axonal connections of the dentate granule cells were altered by exposure to alcohol throughout gestation, but the inputs to the same cells in the dentate gyrus were apparently unaffected.

We next asked whether the timing of the alcohol insult in relation to the stage of development was important for producing the aberrant mossy fibers. A liquid diet containing 35-percent ethanol-derived calories was given to pregnant rats during the first, second, or combined first and second trimester equivalents. Other (neonatal) rats were exposed to alcohol on postnatal days 1 to 10 using the artificial rearing procedure. When examined at adulthood, there was a differential effect on the mossy fiber terminal field due to the timing of the alcohol in the development. The mossy fiber terminal fields in animals exposed to alcohol during either the first or second trimester equivalents did not differ from controls. However, the mossy fiber patterns in rats that were exposed to alcohol throughout gestation (first and second trimester equivalents) were aberrant, similar to results reported previously (West et al. 1981; West and Hodges-Savola 1983). More importantly, the Timm-stained mossy fiber terminal zones in the rats exposed to alcohol during the third trimester equivalent were even more aberrant than those of the animals exposed to alcohol throughout gestation (West and Hamre 1985) (figure 2).

Since the mossy fiber effects were more severe in the rats exposed to alcohol during the third trimester equivalent than in any of the prenatally exposed groups, even though the amount of alcohol given was less, we initially concluded that the third trimester may be a time when the hippocampus is especially sensitive to alcohol. However, in addition to the differences in the developmental timing of the alcohol between the groups, they also differed in the peak blood-alcohol levels; the pups of the third trimester were exposed to higher BACs than the pups exposed in utero. The more recent data from our laboratory indicate that the peak BAC is more important than daily dose in producing CNS-related fetal alcohol effects (Kelly et al., in press; Pierce and West 1986a; Pierce and West 1986b) and suggest that it may have been the higher peak blood-alcohol levels in the animals exposed to alcohol during the third trimester equivalent, rather than differences in temporal vulnerability, that were responsible for the differential effects. This problem, which has yet to be resolved,

illustrates a real difficulty in conducting well-controlled, unconfounded comparisons of the effects of a particular teratogen in different periods of development.

REGIONAL VULNERABILITY

The Timm stain and HRP analysis indicated that different neuronal populations were selectively affected by the alcohol exposure. Another aspect which would indicate differential vulnerability would be cell loss in specific regions of the hippocampus. There are two reasons for investigating the effects of alcohol exposure on neuron number during development. First, comparing the effects of early postnatal alcohol on pyramidal and granule cells in the hippocampal formation would indicate whether neuron populations are more vulnerable to the toxic effects of alcohol after they have been formed (pyramidal cells) or during their period of neurogenesis (granule cells). Second, since it had been hypothesized that the extent of the aberrant infrapyramidal mossy fiber terminal field was a function of the granule cell/pyramidal cell ratio (Gaarskjaer 1978). selective changes in the number of granule and pyramidal cells following early postnatal alcohol exposure would provide additional data related to our previous observations on alterations of the mossy fiber projection.

Given these considerations, we exposed neonatal rats to 9.7 g/kg/day of alcohol in the formula, distributed uniformly over each 24-hour period during postnatal days 4 to 10. Cell counts performed on pups sacrificed on postnatal day 10 revealed that the alcohol treatment resulted in a significant decrease in pyramidal cells, but only in field CA4 (West et al. 1986). However, there was a lo-percent increase in the number of granule cells in the dentate gyrus, which indicates an increase in either neuron production or migration. Together, these changes in neuronal number suggest that an increased ratio of granule cells to pyramidal cells might account for the aberrant mossy fiber terminal fields observed in alcohol-exposed rats, since there may be an overabundance of mossy fiber axons relative to the available synaptic space (West et al. 1981; West and Hodges-Savola 1983; West and Pierce 1984; West and Hamre 1985). Furthermore, these cell count data indicate possible differential susceptibility of different cell types to alcohol. Taken with the results of others (Bauer-Moffett and Altman 1977; Phillips and Cragg 1982), the data suggest that differentiating neurons are more vulnerable to alcohol than proliferating pools of neurons. Clearly, however, this needs to be verified in different areas of the CNS.

From the analysis of both Timm-stained and HRP-labelled afferent projections to the dentate gyrus, it appears that perinatal alcohol exposure affects organization of the terminal fields in the hippocampus proper but not the dentate gyrus. However, as is the case with all negative findings, interpretation of results should be done cautiously; the alcohol may well have effects on a brain region that are not detectable by the measures being used. In fact, as illustrated below, this is exactly the case in the

dentate gyrus when differences are exposed by other experimental manipulations.

Perforant pathway lesions in normal adult rats, which remove most of the input to the outer three-fourths of the dentate molecular layer (Matthews et al. 1976), induce other afferents to the molecular layer to sprout new terminals and reoccupy the deafferented region in a stereotypical manner (Cotman and Nadler 1978). Using a variety of neuroanatomical techniques, we found evidence that the lesion-induced reorganization of both the septal (cholinergic) and the commissural projections to the molecular layer were different in the animals exposed to alcohol throughout gestation from the control animals (Dewey and West 1984; Dewey and West 1985a; West et al. 1984a). Interestingly, the sprouting response of the adult rats treated prenatally with alcohol resembled that which occurs in normally reared rats when the entorhinal lesions are made in preweanling rats rather than in adults (Nadler et al. 1977; Gall and Lynch 1981; West 1984). The enhanced neuroplasticity of the alcohol-treated rats may indicate that some factors involved in lesion-induced reorganization never reached the state of maturation normally found in adult rats. The abnormal sprouting responsiveness does indicate that the dentate gyrus was, in fact, affected by the prenatal alcohol exposure. Therefore, whether or not a region is affected by alcohol exposure during development not only depends on where one looks in the brain but also on how one looks.

CONCLUSION

Our approach to the analysis of the teratogenic effects of alcohol has now evolved to a concentration on anatomical alterations of the hippocampal formation. This has several advantages, not only from a neuromorphological perspective but also for a much broader range of studies of neuroscience and behavior. The large base of knowledge concerning the electrophysiological, neurochemical, and developmental characteristics of the hippocampal formation (Isaacson 1982; Isaacson and Pribram 1986). plus the large amount of literature on its involvement in behavioral processes (O'Keefe and Nadel 1978; Olton et al. 1979; Seifert 1983) provide many directions to correlate neuroanatomical effects of alcohol exposure with these other properties. For example, we have already shown that the condensed exposure to alcohol during the third trimester equivalent produces deficits in development of spatial navigation learning (Goodlett et al., in press), a task known to reauire the normal maturation of hippocampal function (Dyck et al. 1985). Hablitz (1986) has also shown that prenatal alcohol exposure disrupts the normal recurrent inhibition demonstrated in CA1 by paired-pulse stimulation of afferents in the stratum radiatum. Thus, the opportunities exist to analyze the relationship of neuroanatomical, neurophysiological, neurochemical, and behavioral consequences of perinatal alcohol exposure with regard to hippocampal function.

The experimental results described above illustrate a useful approach using an animal model system for the study of the teratogenic effects of a substance of abuse. The research strategy focused on questions that we felt would allow us to extrapolate the answers to the human condition. Our findings have emphasized the problems of direct attempts to estimate the minimum amount of alcohol that is harmful to development of the CNS in humans. What we have tried to do is to ask questions that concern the factors that must be taken into account when the question of how much alcohol is harmful is addressed. These factors are likely to be relevant to the effects of alcohol on human development. From the data discussed in this paper, it is clear that, before the question of how much alcohol is harmful is resolved, the relevance of several factors--the pattern of alcohol exposure, the period of development in which the alcohol exposure occurs. the brain structure and cell types being examined, and the most sensitive parameters to measure--need to be determined. Hopefully, the answers to these questions will eventually lead to an understanding of the mechanism(s) of the teratogenic effects of alcohol on the CNS.

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Endogenous Opioids, Opioid Receptors, and Neuronal Development

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INTRODUCTION

The quintessential question of developmental biology, how do systems composed of many different types of cells that form functional units arise from a single cell, is most keenly posed by the nervous system in which cellular diversification and multicellular organization reaches the pinnacle of complexity. In the rat brain, for example, well over 100 million cells (Smith et al. 1976) are derived and become organized into networks that execute all the sophisticated and subtle capabilities of the nervous system, with most embryologic events taking place within a 5- to 6-week timespan. Needless to say, in the human brain, the magnitude is staggering. The human cerebellum, for instance, contains over 50 billion cells (Zagon et al. 1977). with all these elements largely being derived and organized within a 2- to 3-year period. Research efforts to delineate the major morphogenetic processes (cellular proliferation, migration, differentiation, growth, and death) have been revealing. One avenue of investigation, elucidation of the role of endogenous opioid systems, i.e., endogenous opioids and opioid receptors, has been particularly promising and has provided new and exciting concepts in developmental biology and neuroplasticity. The intention of this chapter is to outline some of the historical background, principles, and evidence regarding endogenous opioid and opioid receptor control of neurodevelopment and to discuss briefly the implications of these findings to normal and abnormal brain development.

HISTORICAL BACKGROUND, PRINCIPLES. AND EVIDENCE

The roots of the discovery that endogenous opioid systems regulate neurobiological development (Zagon and McLaughlin 1983a; Zagon and McLaughlin 1983b; Zagon and McLaughlin 1984a) lie not only in the research interests of this laboratory in perinatal drug abuse (Zagon 1985; Zagon et al. 1982; Zagon et al. 1984) but in the revelation that opioid receptors (Simon et al. 1973; Pert and Snyder 1973; Terenius 1973) and endogenous opioids (Hughes 1975) are present in neural tissues. Intrigued by research findings

that endogenous opioid systems exert a marked influence on the growth of neural tumors (Zagon and McLaughlin 1983c). we raised the question as to whether endogenous opioids and opioid receptors played a role in neuro-ontogeny. We utilized opioid antagonists to interfere with the interaction of endogenous opioids and opioid receptors in the developing brain. The use of opioid antagonists rather than opioid agonists was motivated by our knowledge that the half-lives of endogenous opioid agonists are as little as a few minutes and that interpretation of the results from these experiments could be extremely complex, since it would be difficult to distinguish between action and reaction following opioid agonist administration. In contrast, the opioid antagonist paradigm allows one to monitor precisely the duration of opioid receptor blockade by measuring nociception, e.g., hot plate test, following a challenge with opioid agonists such as morphine.

In the first series of experiments, naltrexone, a potent opioid antagonist, was administered on a chronic basis to preweaning rats (Zagon and McLaughlin 1983a). Preweanina develounent in the rat (birth to day 21) is characterized by explosive body and brain growth and serves as a good model to examine the influence of various agents on developmental events. At 21 days, body, brain, and cerebellar weights of rats receiving daily injections of naltrexone at a dosage (50 mg/kg) that blocked morphine-induced analgesia 24 hours/day were 18 percent, 11 percent, and 5 percent greater than control weights, respectively. In addition, morphometric analysis of the cerebrum revealed a somatosensory cortex that was 18 percent thicker than that of control animals. The cerebellum of naltrexone-treated rats was 41 percent larger in total area and contained 70 percent more glial cells and 30 percent more internal granule neurons. Neurons derived prenatally, such as Purkinje cells, were unaffected in terms of cell number by opioid antagonist treatment. The results of these early experiments provided evidence that endogenous opioids and opioid receptors normally interact during brain development and suggest that endogenous opioid systems may play an integral role in cell proliferation, migration, and differentiation.

Further information about neurodevelopment and endogenous opioid systems was revealed in experiments contrasting a dosage (50 mg/kg naltrexone) of opioid antagonist that blocked the receptor for an entire 24-hour injection period with a dosage (1 mg/kg naltrexone) that blocked the opioid receptor for a short time each day, i.e., 4 hours per injection period (Zagon and McLaughlin 1983b). When drug injections began at birth, rats injected with 50 mg/kg naltrexone showed increases in body weight over controls by 3 days of age, with significant increases in body weight recorded throughout the preweaning period, i.e., days 3, 6, 10, 15, and 21. Rats injected with 1 mg/kg naltrexone were subnormal in weight during the preweaning period, with statistically reliable reductions noted on days 6, 10, 15, and 21. Consonant with the data on body weights, the effect of naltrexone on organ growth was dose related. The wet weights of brain, heart, kidneys, liver, and skeletal muscle were 11 to 32 percent more than controls for rats in the 50 mg/kg

group but 5 to 24 percent below normal for animals given 1 mg/kg naltrexone. Calculation of relative organ weights, i.e., organ to body weight ratios, indicated that the changes in organ wet weights of naltrexone-treated animals were generally proportional to that of body weights with only two exceptions. In the 50 mg/kg naltrexone group, brain weight was significantly less affected than body weight, whereas the weight of skeletal muscle was markedly more affected than body weight.

In these experiments, the timing of the appearance of certain physical characteristics, i.e., hair covering, incisor eruption, ear opening, eye opening, was evaluated. Animals in the 50 mg/kg naltrexone group exhibited a significantly earlier appearance of eye opening than controls, whereas the 1 mg/kg naltrexone group showed a significant retardation in the proportion of animals with ear and eye opening on the median day. Measurement as to the initial appearance of walking, a milestone in behavioral ontogeny, showed that the 50 mg/kg naltrexone group was able to walk between days 8 to 12, while the animals in the 1 mg/kg naltrexone group walked between days 13 to 15; the range for control subjects was 10 to 13 days. Furthermore, at the age when only half (50 percent) of the controls were able to walk, no rats in the 1 mg/kg naltrexone group could walk, and all (100 percent) of the animals in the 50 mg/kg naltrexone group could walk. These results indicated that opioid antagonist administration could markedly alter the course of somatic and neurobiological development, with the direction of effects dependent on dosage. The dosages of 1 and 50 mg/kg naltrexone mediating these actions were remarkably low (0.05 to 2.5 percent, respectively, of the LD_{50}). However, both dosages were more than sufficient to antagonize morphine-induced analgesia. Therefore, complete opioid receptor blockade each day stimulates development, whereas intermittent daily receptor blockade inhibits growth.

Although opioid challenge experiments revealed that the 50 mg/kg dosage blocked the opioid receptor for 24 hours/day, and a dosage of 1 mg/kg naltrexone blocked the receptor for 4 to 6 hours/day, the relationships among growth processes, drug dosages, and the pharmacological properties of naltrexone, e.g., ability to block the opioid receptor, required elucidation. A series of experiments was designed to explore this question (Zagon and McLaughlin 1984a). In the first study, groups of neonatal rats received daily injections of either 0.1, 1, 10, 20, 50, or 100 mg/kg naltrexone or sterile water, with body and brain weights sampled at weaning. Daily injections of 0.1 to 50 mg/kg naltrexone had no effect on infant viability during the preweaning period. However, daily injections of 100 mg/kg naltrexone resulted in a 40-percent mortality rate. The effect of naltrexone on body weight was clearly dependent on dosage. Animals receiving 0.1, 1, or 10 mg/kg naltrexone weighed 11 to 13 percent less than 21-day-old control rats, whereas animals receiving 20, 50, or 100 mg/kg naltrexone weighed 16 to 22 percent more than controls. Comparison of the 0.1, 1, and 10 mg/kg naltrexone groups, as well as the 20, 50, and 100 mg/kg naltrexone groups, revealed no between-group

differences within either set of dosages. The effects of naltrexone on brain weight were also dependent on dosage. Rats in the 0.1 and 1 mg/kg naltrexone groups had brains weighing markedly less (5 percent) than those of control animals. The brain weights of rats in the 10 mg/kg naltrexone group also were less than controls, but the difference was not statistically significant. Animals in the 20, 50, and 100 mg/kg naltrexone groups had brain weights that were increased 6 to 13 percent from control values. To examine whether the marked biphasic effect of naltrexone on growth was related to the pharmacological action of opioid receptor blockade induced by naltrexone, 21-day-old rats receiving 1, 10, 20, or 50 mg/kg naltrexone were challenged with morphine. The results show that a dosage of 1 or 10 mg/kg naltrexone blocked the antinociceptive effect of morphine for 4 to 6 hours and 6 to 12 hours, respectively. Dosages of 20 or 50 mg/kg naltrexone were effective in blocking the opioid receptor for an entire 24-hour injection period.

In order to investigate further whether body and brain development were related to the pharmacological action of naltrexone in blocking the opioid receptor, as well as to explore whether low and high dosages of naltrexone produced growth effects because of interaction with different populations of receptors, a second set of experiments was performed. Growth and nociceptive response were monitored in rats given 3 mg/kg naltrexone three times daily, a dose which blocked the opioid receptor for an entire 24-hour period. Increases of 31 percent and 10 percent in body and brain weights, respectively, were found compared to control levels. However, once daily injections of 9 mg/kg naltrexone (the cumulative dosage of three daily injections of 3 mg/kg), a dosage that blocked the opioid receptor for 6 to 12 hours/day, resulted in animals with a C-percent decrease in body weight and no statistical change in brain weight. These results clearly demonstrated that the duration of opioid receptor blockade governs somatic and neurobiological development.

In view of the changes in body and brain growth, along with the provocative finding concerning the timetable for walking, we investigated the effects of interruption of endogenous opioid/opioid receptor interaction by opioid antagonists on neurobehavioral ontogeny (Zagon and McLaughlin 1985). The development of spontaneous motor and sensorimotor behaviors of preweaning rats, as well as ambulation, emotionality, and nociception at weaning (day 21) were studied in rats given chronic administration of 1 or 50 mg/kg naltrexone from birth to day 21. The age at which a specific spontaneous motor behavior or performance initially appeared and the age at which 100 percent of the animals demonstrated a particular behavior were accelerated in animals given 50 mg/kg naltrexone but were delayed in rats injected with 1 mg/kg naltrexone. In general, ambulation, emotionality, and nociceptive responses were not affected by naltrexone treatment, although the frequency of face washing in both naltrexone groups and activity cage performance in the 50 mg/kg naltrexone group deviated from control levels. Observations of head-shake and wet-dog-shake behaviors in

naltrexone-treated animals at 2 hours and 10 hours after drug injection were similar to controls with the exception of an abnormal increase in the 1 mg/kg naltrexone group at 10 hours. These results imply that endogenous opioid systems play a role in regulating neurobehavioral development. Further study will be needed to learn whether these changes are a consequence of the somatic and morphological alterations known to occur with naltrexone administration, or if the timetable of behavioral ontogeny is dictated by endogenous opioid/opioid receptor interaction.

A series of histological and morphometric studies was conducted to establish more fully the role of endogenous opioid systems in regulating brain development (Z gon and McLaughlin 1986a; Zagon and McLaughlin 1986b). In the irst study (Zagon and McLaughlin 1986a), the developing cerebella of 21-day-old rats given a temporary (1 mg/kg naltrexone) or complete (50 mg/kg naltrexone) blockade of opioid receptors th oughout the first 3 weeks of postnatal life were investigated. I general, 50 mg/kg naltrexone had a stimulatory action on cerebellar development, whereas 1 mg/kg naltrexone had an inhibitory influence. Both sexes were affected comparably. Limits to the ability of naltrexone to modulate cerebellar ontogeny were noted, with more latitude existing toward growth enhancement than impairment. The temporal course of neurogenesis was not delayed in naltrexone-treated rats; abbreviation of the timetable of neuro-ontogeny remains to be explored. Apparently, the events that transpired over the preweaning period were dramatically affected. The most notable effects in 1 mg/kg naltrexone rats were marked decreases in dimensions of cerebellar area, the content of internal granule neurons, and cellular and tissue differentiation. Characteristics of the 50 mg/kg naltrexone group included increases in dimensions of cerebellar area, neural cell number, content, and size, and structural changes consistent with acceleration in growth and differentiation. Naltrexone influenced both neurons and glia, but only neural cells still being generated during the first 21 days after birth were altered in regard to quantity.

In a companion study (Zagon and McLaughlin 1986b) on opioid antagonist modulation of cerebral and hippocampal development, the same experimental paradigm was utilized. As with the cerebellum, 50 mg/kg naltrexone had a stimulatory effect on growth, whereas 1 mg/kg naltrexone had an inhibitory effect, with both males and females being affected comparably. Opioid antagonist action was especially directed at cellular and tissue differentiation, with marked changes in macroscopic dimensions of area and histotypic organization observed in the cerebrum. A prominent effect on the cerebrum of the 1 mg/kg naltrexone group was a substantial increase in packing density of the neural cells, reflecting a reduced area for accommodating neural elements. Changes in the hippocampus were largely restricted to the 1 mg/kg group. However, the number of granule cells was increased in the dentate gyrus of the 50 mg/kg group, suggesting that opioid receptor blockade affects cell types undergoing postnatal proliferation.

As in the cerebellum, cellular elements derived prior to naltrexone treatment, e.g., pyramidal neurons, were capable of being influenced in only their capacity to differentiate.

It is possible that the establishment of neuronal organization and intricacy in the developing brain is dependent on endogenous opioid/opioid receptor interactions. To address the role of endogenous opioids in neuroplasticity, developing neurons in the cerebral cortex, hippocampus, and cerebellum were structurally examined in lo-day-old rats subjected to continuous opioid receptor blockade, i.e., 50 mg/kg naltrexone (Hauser et al., submitted for publication). The lengths of oblique dendrites of pyramidal cells in the cerebral cortex and basilar dendrites of the hippocampus were increased 136 percent and 52 percent, respectively, from controls, whereas the concentrations of spines in these cells were increased 183 percent and 69 percent, respectively. Dendritic area and the number of spiny branches of cerebellar Purkinje neurons were 43 percent greater than controls, and spine concentration of granule cells in the dentate gyrus was increased 76 percent. Thus, endogenous opioid systems appear to play a profound role in neuronal growth and the expression of dendritic spines, suggesting that the developing nervous system is plastic and modifiable by endogenous opioid/opioid receptor interactions.

In view of the enormous changes in growth induced by opioid antagonists, opioid-antagonist-associated alterations in the hormonal milieu and nutrition should be discussed. The possibility that endogenous opioid control of developmental expression is an indirect effect of hormonal changes, such as that of thyroid hormones, pituitary hormones, and adrenal and testicular androgens, is difficult to support. For example, none of these hormones has been demonstrated to make a normal animal larger; they can only bring a hormonally deficient animal to normal levels (Zamenhoff (1971). Moreover, the developmental actions recorded with opioid antagonists are not comparable to morphological studies previously conducted. Furthermore, administration of opioid antagonists such as naloxone and naltrexone to rats has been reported to cause no alterations in levels of physiological growth hormone or insulin secretions (Tannenbaum et al. 1979). Insofar as nutrition is concerned, the possibility that opioid antagonists (perhaps mediated by endogenous opioid systems) alter food consumption. and hence growth, warrants consideration. Investigations of the effects of acute injections of naltrexone on milk intake in preweaning rats has been undertaken by Aroyewun and Barr (1982). Their results show no differences in milk intake between lo-day-old experimental animals receiving 10, 30, or 50 mg/kg naltrexone or saline-injected rats. At 14 days of age, only the 50 mg/kg naltrexone group differed from controls, with these animals consuming markedly less than control pups. Thus, nutritional deficits should not have been involved in the decreased body weights observed in rats given 10 mg/kg or less of naltrexone. As for the 20 to 100 mg/kg dosages of naltrexone, these animals weighed more than controls, even though some dosages (50 mg/kg) may have led to reduced milk consumption. Moreover, the stimulatory and inhibitory actions of

naltrexone on growth were observed by 3 and 5 days of postnatal life, respectively, a time well before any problems in milk consumption were detectable by Aroyewun and Barr. To investigate further the possibility that inadequate nutrition led to lower body weight, a comparison of the brain weights of 1 mg/kg naltrexone animals to a group of control rats matched for body weight was conducted. In spite of the brain-sparing effect classically observed with nutritional deficits, we found that 1 mg/kg naltrexone-treated pups at 21 days had significantly smaller brains than those of control animals matched for weight. In other words, the brains were not "spared" in the naltrexone group, suggesting that the actions of naltrexone may not be nutritionally related. As for the possibility that the stimulatory effect of naltrexone was due to greater food consumption, overnutrition during the preweaning period cannot increase growth beyond normal limits (Wurtman and Miller 1976). Thus, alterations in nutrition or hormones do not appear to be a primary locus of opioid-antagonist-induced growth regulation.

The work using the opioid antagonist paradigm casts light on the role of endogenous opioid systems in growth. It would appear that endogenous opioids have a normal and important interaction with opioid receptors during nerodevelopment. Abolishment of this interfacing, either with a high dose of naltrexone or multiple daily injections with a low dose, produces an animal with an accelerated pattern of growth; cell proliferation, migration, differentiation, and growth are affected. A temporary blockade of opioid receptors each day seems to retard growth. Although more work needs to be performed, it is known that administration of opioid antagonists causes an increase in opioid receptors, a supersensitivity to opioid agonists, and an increase in plasma levels of endogenous opioids (Bardo et al. 1983; Recant et al. 1980). Thus, the growth inhibition observed with low dosages of naltrexone administered once daily may be the result of interaction of basal OF elevated levels of endogenous opioids with an increased number of receptors, producing an enhanced reaction during the daily interval when opioid antagonist is no longer present.

Evidence for direct involvement of endogenous opioids and opioid receptors in growth comes from a number of other quarters. Experiments examining the effects of exogenous opioids on neuroblastoma cells in culture reveal that opioid agonists inhibit growth in a dose-response, stereospecific, and naloxone-reversible manner (Zagon and McLaughlin 1984b; McLaughlin and Zagon 1984). Moreover, endogenous opioids such as methionine enkephalin administered to neuroblastoma cells in culture are also inhibitory to growth in a naloxone-reversible fashion. The question of whether endogenous opioid systems act directly under in vivo conditions also should be considered. It is well known that opioid receptors are associated with developing neural cells (Gibson and Vernadakis 1981; Spain et al. 1985) and that endogenous opioids are present in growing nervous tissue (Maseda et al. 1983). For example, ^{3}H-methionine enkephalin and H-naloxone receptor binding have been

detected in the developing cerebellum (Tsang and Ng 1980; Tsang et al. 1982a; Tsang et al. 1982b), with the highest levels recorded within the first few days of life, declining to low levels shortly thereafter. ß-endorphin and enkephalin levels in the cerebellum were also found to reach their highest levels in the first post-partum week and to decline subsequently to low levels (Maseda et al. 1983; Tsang et al. 1982b). Recently, the distribution of enkephalin in the cerebellum was examined by immunocytochemistry (Zagon et al. 1985). Methionine and leucine enkephalin were found to be concentrated in the external aerminal cells of the 10-day-old rat cerebellum and localized throughout the cortical cytoplasm. Enkephalin was not detected by immunocytochemistry in differentiated neural cells originating from this iayer, e.g.; internal granule neurons. Further work in our laboratory has now detected enkephalin immnunoreactivity within the developing germinal cells using immunoelectron microscopic procedures. This activity was not recorded in the cell nucleus but was apparent throughout the cortical cytoplasm, often being associated with mitochondria (Prouty et al., unpublished observations).

Thus, the results of studies using the opioid antagonist paradigm, as well as investigations of endogenous opioids and opioid receptors in developing systems, reveal that the endogenous opioids appear to regulate neurodevelopment by an inhibitory mechanism. The endogenous opioids seem to be extremely active in modulating growth, since blockade of endogenous opioid/opioid receptor interaction results in altered growth within a short period of time.

A number of major questions need to be addressed at this juncture. Two of the most pressing are: What peptides and receptors are involved in neurodevelopment? How do neuropeptides and receptors interact at the cellular and molecular levels to affect their regulation of growth? Some information does exist as to the type of opioid receptor that may be involved in neuro-ontogeny. Studies using ß-funaltrexamine (B-FNA), a highly selective and irreversible µ opioid receptor antagonist, have been conducted in preweaning rats (Zagon and McLaughlin 1986c). Animals given B-FNA did not differ from controls in body weight, brain and cerebellar weight, macroscopic dimension of the brain, area of the cerebellum, or organ weight. The dosage of ß-FNA employed (5 mg/kg) blocked morphine-induced analgesia (2 mg/kg morphine sulfate) for each injection period (48 hours). In contrast to B-FNA treatment, rats given naltrexone (50 mg/kg) in a regimen which completely blocked the opioid receptor throughout ontogeny exhibited marked increases in somatic and neurobiological growth. These results suggest that, in and of themselves, µ receptors selectively antagonized by B-FNA do not play an important part in regulating development. Further studies with specific opioid agonists and antagonists in this regard will be valuable.

CONCLUSIONS AND IMPLICATIONS

In view of the evidence briefly presented in this chapter, endogenous opioid systems are extremely important in regulating the

development of the nervous system. These findings hold great promise in terms of understanding normal and abnormal development and for the design of experiments to probe growth. One of the critical issues in this regard is whether dysfunction of the endogenous opioid systems, be it problems in opioid receptors and/or endogenous opioids, is involved in problems of development of the nervous system in children. More information about endogenous opioids and opioid receptors may contribute to the potential for therapeutic intervention in growth-related abnormalities, especially those targeting neural development. Knowledge about endogenous opioids and opioid receptors in regard to neurobiological development may also hold keys to unraveling the mechanisms involved in perinatal opioid exposure. It has been estimated that up to 230,000 children have already been perinatally exposed to opioids such as heroin, methadone, and morphine (Zagon et al. 1984; Zagon 1985). These children often display growth impairment along with a constellation of neurobehavioral irregularities, including abnormalities in motor skills, learnfng, and interpersonal relationships. Little is known about the etiology and pathogenesis of the perinatal opioid syndrome. It is feasible that exogenous opioids are inhibiting development by the same mechanism used by endogenous opioids to control growth normally, and more research into this area could prove *to* be an important *source* of clinical prevention and treatment.

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Endocrine Consequences of Perinatal Methadone Exposure

Cynthia Kuhn, Lisa Bero, Diane Ignar, Scott Lurie, and Elizabeth Field

INTRODUCTION

The response of the developing nervous system to chronic perturbation, either pharmacologic or behavioral, is a vital area in the understanding of neural adaptation in general. Although the immature nervous system is generally viewed as more vulnerable to insult, it simultaneously shows the potential to recover from damage that would be devastating to adults. The ability of young patients to recover from central nervous system (CNS) insults is a well-known proof of this precept. The adverse consequences of exposing the developing nervous system to alcohol, cocaine, or other CNS-active drugs provide a marked contrast, in producing notable and permanent disruption of CNS function. Because our understanding of neural development is so poor, it is very difficult to predict the outcome of exposing the developing nervous system to chronic pharmacologic challenge. The goal of research in this area is to determine which processes are relatively at risk and which are relatively protected in the immature organism compared to the adult. The experimental model used in this study of the problem is an examination of the effect of chronic opiate administration on endogenous opioid control of developing neuroendocrine function, and its perturbation by chronic exposure to opioids. This project has almost become a case study in the difficulties and rewards of understanding the capacity of the immature nervous system to adapt to a chronic pharmacologic stimulus.

BACKGROUND

Review of the Literature

This work was stimulated by the significant literature suggesting that offspring of women who use opiate analgesics throughout pregnancy are addicted and undergo withdrawal at birth. These children exhibit low birth weight and delays of somatic and neurobehavioral development (Zagon et al, 1982; Zagon and McLaughlin 1984; Sonderegger and Zimmerman 1979; Hans et al. 1984). The recent explosion of information about endogenous opioids and their

potential role in regulation of developmental events has provided potential physiological mechanisms to explain these clinical findings and has stimulated interest in the field.

It is possible that disrupted endocrine function might contribute to the disturbances of somatic and behavioral development following perinatal opiate addiction, since endogenous opioid systems play a role in neuroendocrine regulation in adult animals. Anecdotal reports of altered hormone levels in addicted infants and in animals exposed to opiates during development include transient elevations of thyroxine and prolactin (PRL) at birth, as well as changes in thyroid, glucocorticoid, and ovarian responsivity to challenge later in life (Glass 1982; Rosenfeld et al. 1981; Jhaveri et al. 1980; Litto et al. 1983; Sonderegger and Zimmerman 1978; Bakke et al. 1973). These changes could result from morphologic damage to specific populations of neurons during development (a classical teratogenic effect); alternatively, they might simply reflect adaptations in the neural networks involved in opioid-induced changes in hormone secretion which are somewhat different from the adaptations which occur in adults following similar treatments. This distinction is central to all problems in developmental neurotoxicology and is particularly important if the disrupting agent, like the opiates, has marked effects on the adult nervous system which resemble, at least in part, those caused in the immature brain. The experimental model developed in these studies has proven particularly useful in addressing this question, and the results support the notion that at least some of the endocrine consequences of perinatal opiate exposure represent the latter process.

Studies of developmental effects of perinatal opiate exposure often involve chronic administration of opiates during gestational and/or postnatal development in treatment regimens that have been designed more to resemble clinical exposure of developing humans than to address specific mechanistic questions. As a result, there is considerable phehomenologic literature reporting changes in opiate sensitivity in adult animals following neonatal exposure (mainly persistent tolerance although occasionally enhanced activity of opiates after perinatal exposure (Kirby et al. 1982; Zimmerman et al. 1974; Thompson and Zagon 1981; Zagon and McLaughlin 1980; Hovious and Peters 1984)), but there has been little systematic comparison between the responses of neonatal and adult animals to similar chronic treatment regimens. Furthermore, there are few studies in which potential biological concomitants, such as changing receptor numbers, have been measured following chronic developmental administration of opioid agonists or antagonists, and these few studies have yielded inconsistent results: Some investigators report increased receptor number and others, decreased receptor number (Bardo et al. 1983; Bardo et al. 1982; Tsang and Ng 1980; Handelman and Quirion 1983).

MODEL FOR STUDY OF PERINATAL OPIATE EFFECTS

One major source for such disagreement has been the diversity of treatment paradigms, behavioral end points, and biochemical assessments. Also, many of these studies were conducted a number of years ago, before the multiplicity of endogenous opioid systems had been defined. Therefore, determinations of whole brain opiate receptor numbers were made from averaged results of disparate endogenous opioid systems with different ontogenetic development and different responses to pharmacologic challenge. Assessing opiate action in immature animals presents an additional problem in these studies. Many opioid effects, including analgesia, control of respiration, and locomotor effects, cannot be measured conveniently in immature animals or act through neural pathways that mature after a major part of the treatment paradigm is completed (and animals are already tolerant to the effects of administered opiates). This problem of behavioral evaluation is implicit in all studies of developing nervous system function.

A strategy that is very useful in circumventing this difficulty is to use neuroendocrine function, specifically, secretion of anterior pituitary hormones, as a model behavior. Hormone secretion can be measured in animals too young for more sophisticated behavioral assessments, i.e., during the first postnatal week. For studying endogenous opioid systems, this strategy is particularly useful, because opioid effects on hormone secretion are mediated mainly by fairly well-defined neural networks. Furthermore, opioid control of hormone secretion matures early in comparison to other neural controls. For example, morphine causes profound stimulation of growth hormone (GH) secretion as early as postnatal day 5 in the rat, while other pharmacologic stimuli, such as pentobarbital and the α-adrenergic agonist clonidine, are not effective until weaning or later (Kuhn and Schanberg 1981). Similarly early ontogeny of opioid effects has been described in the fetal sheep, an elegant model of neuroendocrine ontogeny which has been studied in detail (Gluckman et al. 1980).

In this study, we have been investigating systematically the pattern of endocrine responses to various acute and chronic opioid manipulations. These responses include studies of both basal hormone levels and hormone secretion following pharmacologic challenge. We have used the rat for these studies, because many aspects of opioid regulation of neuroendocrine function, as well as perinatal opioid addiction, resemble phenomena observed in humans. However, this model, like any other, has several drawbacks, including the possible role of nutritional and physiologic variables in endocrine function, and all effects of perinatal opiate manipulations must be interpreted with these considerations in mind. In many of our chronic studies, methadone has been used for the direct clinical relevance of the finding. However, there were only minor differences among the endocrine responses elicited by different opiate alkaloids.

MULTIPLE ENDOCRINE RESPONSES TO OPIATES IN ADULTS

In adult rats, the endocrine effects of opiate agonists have been well characterized (figure 1): Opiates increase serum levels of GH, PRL, adrenocorticotropic hormone (ACTH). and corticosterone (CS), and decrease thyroid-stimulating hormone (TSH) secretion

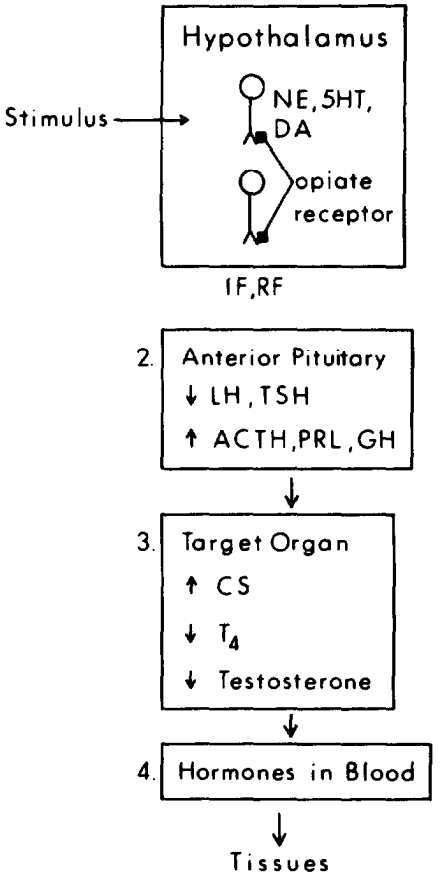

FIGURE 1. *Model of opioid effects on anterior pituitary hormone secretion*

(Millan and Herz 1985; Howlett and Rees 1986; Meites et al. 1979). Variable decreases in luteinizing hormone (LH) secretion are reported in these studies, depending upon the age and sex of the animal. The most sensitive indicator of opioid involvement in regulation of LH secretion is the pronounced elevation of LH observed following administration of the opioid antagonist naloxone. Similar results follow the administration of the opiate agonist

methadone (figure 2). The development of tolerance to these different endocrine effects of opiates in adults have been studied in the past but only using a single treatment paradigm and a single

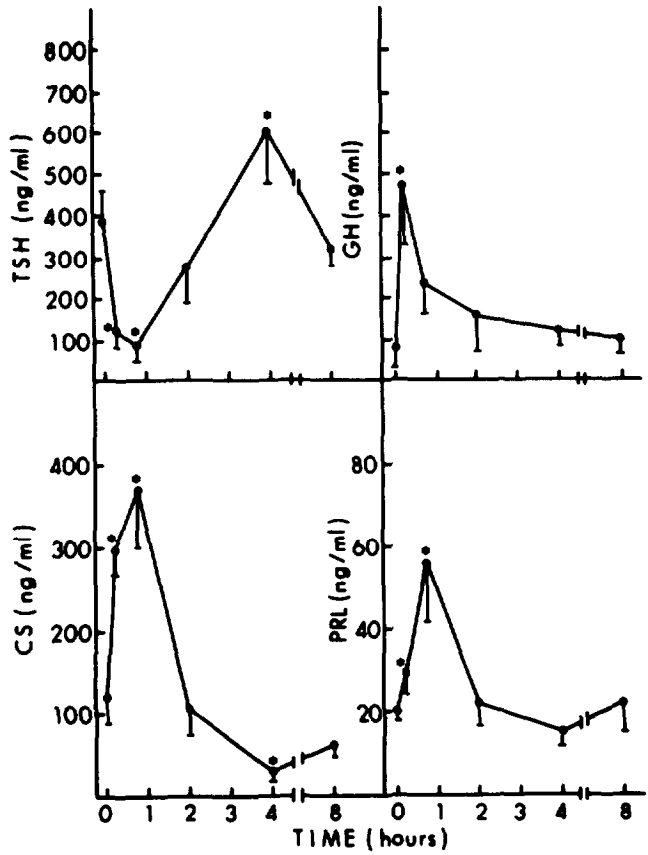

FIGURE 2. *Effect of methadone (2.5 mg/kg) on serum hormone levels in adult male rats*

KEY: * Indicates statistically different from time-matched control, p<.05 or better.

NOTE: Animals were treated with methadone and killed at various times after treatment, and hormone levels were then determined. Results are expressed as man ± SEM. Saline-injected controls were killed at each time point. As control values did not differ from each other, values were pooled and shown at t=0. However, appropriate time-matched controls were used for statistical evaluation. N=6-8 in each group.

endocrine measure (Cicero et al. 1975; Paroli and Melchiorri 1961; Kokka et al. 1973; Muraki and Tokunaga 1978; Deyo et al. 1980; Eisenberg and Sparber 1979; Miodustewski et al. 1982). Therefore, it is difficult to compare the development of tolerance at different sites. The comparison of multiple endocrine responses has proved extremely useful in understanding opiate tolerance in adult and developing animals.

When adult rats were treated with methadone chronically and the pattern of endocrine response to a challenge dose of methadone was assessed, the results demonstrated clearly that tolerance development is quite specific for different endocrine systems. For example, the development of tolerance to opiate-induced changes in CS is observed following a low-dose treatment regimen (methadone 5 mg/kg/day for 20 days) (figure 3). Following treatment with increasing dose regimens of methadone, changes in LH, TSH, PRL, and GH follow in sequence, although the effects of methadone tolerance on GH and PRL secretion develop slowly and occur only with extremely high dose regimens. This specificity offers an opportunity for studying differential tolerance development in various endogenous opioid neurons using a single model system. For example, recent delineation of the roles of μ, κ, and δ opiate receptors has offered an avenue for understanding these patterns of response in both adult and developing animals. Another important criterion of tolerance development in endocrine systems is the specificity for the effects of opioids. For example, CS responses to opiates are eliminated following even short treatment regimens that do not influence responses to agents which act on different neural regulation of CS secretion, such as CS responses to the acetylcholinesterase inhibitor physostigmine or the dopamine ß hydroxylase inhibitor diethyldithiocarbamate (DDC) (figure 4). These findings suggest that tolerance does not develop through adaptation at the anterior pituitary or the end organ, an important observation if endocrine function is to be used as a model of neuronal tolerance.

In neonatal rats, endocrine responses to opioids resemble qualitatively those observed in adults. As early as postnatal day 5, methadone or morphine increase GH, PRL, and CS and decrease TSH secretion (Kuhn and Bartolome 1983), and a profound opioid inhibition of LH secretion is indicated in female rats by the large increases in LH secretion which follow naloxone administration (Cicero et al. 1986). Administration of endogenous opioids, including ß-endorphin, various enkephalin derivatives, and dynorphin, elicits changes in endocrine function identical to those caused by administration of methadone. For example, ß-endorphin administration to a 5-day-old rat pup increases GH, PRL, and CS and decreases TSH secretion significantly (figure 5).

However, several characteristics of the endocrine response in neonates differ significantly from the response observed in adults, and these differences represent a common and important occurrence that influences interpretation of chronic studies.

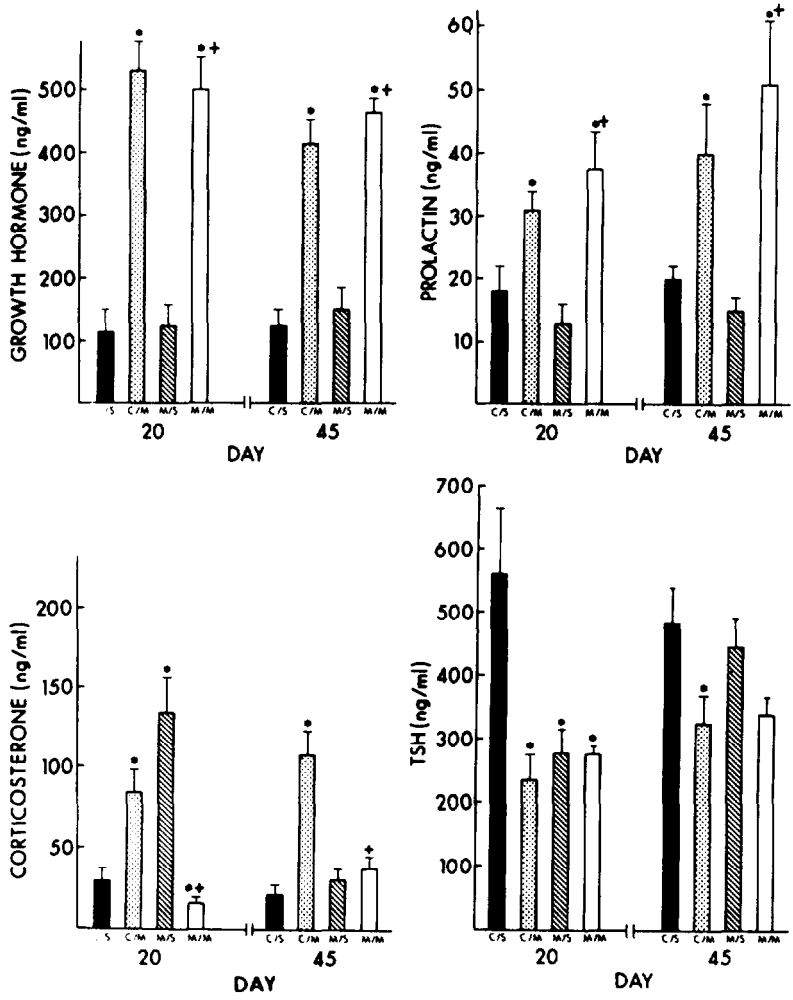

FIGURE 3. *Endocrine response to a challenge dose of methadone following chronic treatment of adult male rats*

KEY: C/S (black bar) = chronic saline, acute saline; C/M (stippled bar) = chronic saline, acute methadone; M/S (striped bar) = chronic methadone, acute saline; M/M (clear bar) = chronic methadone, acute methadone; * indicates significantly different from C/S control, p<.05 or batter; + indicates statistically different from M/S control, p<.05 or better.

NOTE: Animals were treated with saline or methadone (5 mg/kg/day) for 20 days and given a challenge dose of methadone at the end of the treatment regimen (or 2 reeks later on day 45), killed, and their hormone levels determined. Results are expressed as mean ± SEM. N=6-8 In each group.

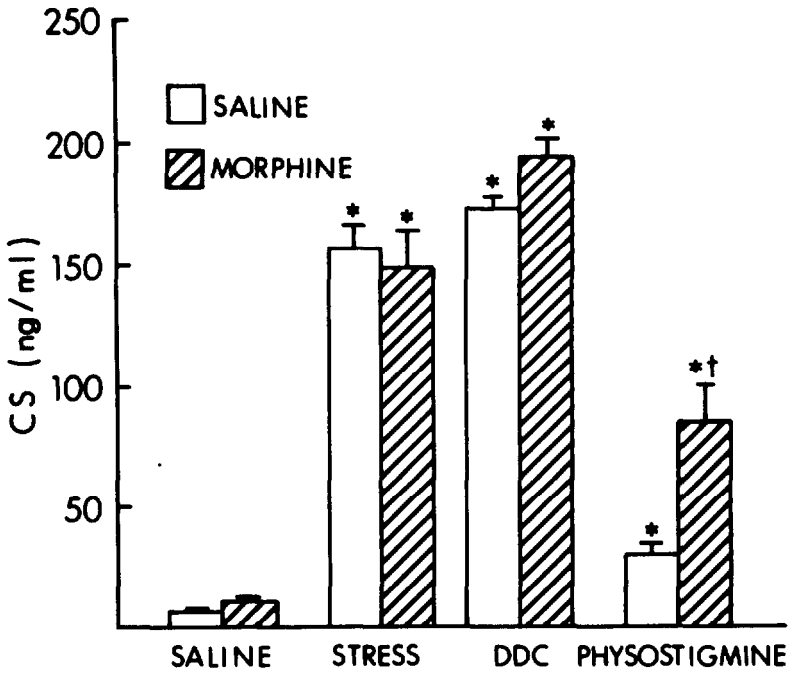

FIGURE 4. *Effect of increasing morphine injections (5 mg/kg, b.i.d., increasing by 5 mg/kg/injection over 5 days) on CS response to saline, morphine, physostigmine, or DDC*

KEY: * Indicates statistically different from chronic saline control, $p<.05$ or better; † indicates statistically different from chronic morphine control, $p<.05$.

NOTE: Animals were treated chronically, withdrawn for 36 hours, then treated with saline, morphine (10 mg/kg), physostigmine (0.5 mg/kg), or DDC (100 mg/kg). Al I drug pretreatments were given 45 minutes before killing, except DDC which was given 4 hours before killing. Results are expressed as mean ± SEM. N=8-10 in each group.

First, the responses last significantly longer, in part because of the slowed elimination of opiates from neonatal rats (figure 6) (Shah and Donald 1978). This phenomenon obviously must influence the choice of treatment regimen in the pup, as administration of identical doses to pups and adults can result in relatively higher drug exposure for the pups. However, many endocrine responses are smaller, in part because pituitary stores and responsiveness to releasing factors as well as hormonal feedback effects on secretion tend to be somewhat blunted during the early postnatal

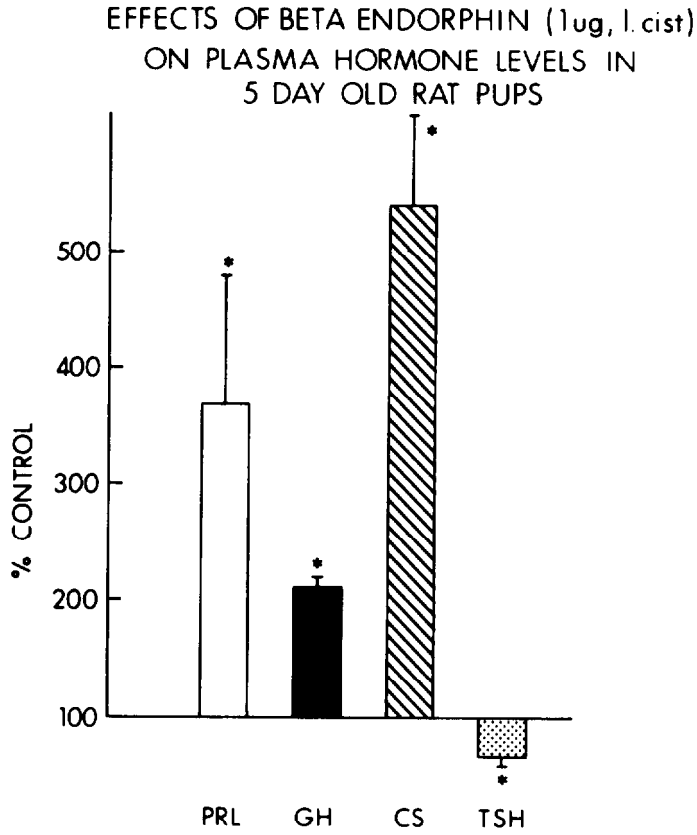

FIGURE 5. *Endocrine response to ß-endorphin in neonatal rats*

KEY: * Indicates statistically different from saline-Injected control, p<.05 or better.

NOTE: Five-day-old rat pups were Injected intracerebroventricularly with saline or ß-endorphln (0.1 µg) and killed at various times after injectlon. After the animals were killed, hormone levels were determined. Results are expressed as percent control ± SEM. N=6-8 In each group.

period in rats for GH, PRL, and TSH (Fisher et al. 1977; Goodyer 1981). Furthermore, the number of opioid receptors is much lower, thereby limiting the maximal possible response attainable (Wohltmann et al. 1982; Tsang et al. 1982a; Tsang et al. 1982b; Kent et al. 1982; Patey et al. 1982; Tsang and Ng 1982). Therefore, pups tend to be somewhat less responsive despite the presence of greater drug levels.

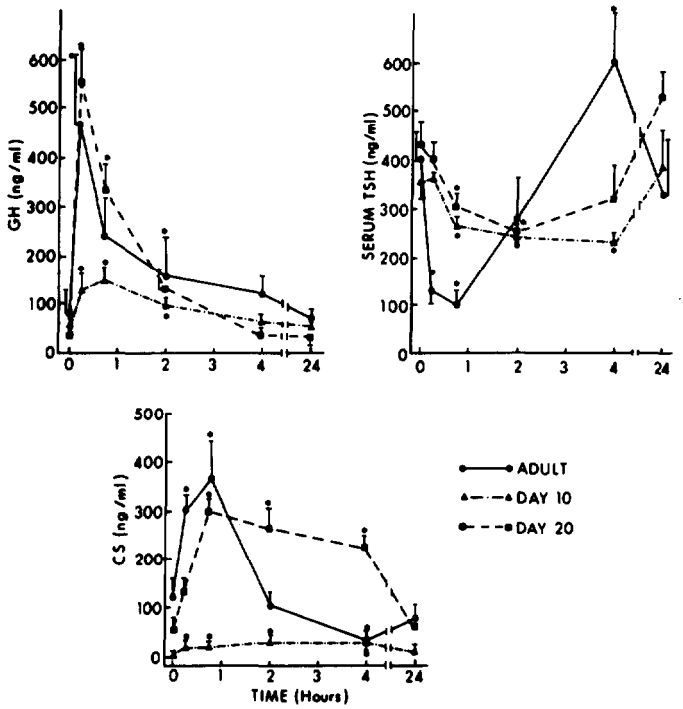

FIGURE 6. *Effect of methadone (2.5 mg/kg) on serum hormone levels at different postnatal ages*

KEY: * Indicates statistically different ($p<.05$ or better) than time-matched control.

NOTE: Ten-day-old, twenty-day-old, and sixty-day-old (adult) animals were treated with saline or methadone and killed at various times after drug injection; then serum hormone levels were measured. Results are expressed as described in figure 2. N=8 or more In each group.

POSTNATAL ACUTE OPIATE CHALLENGE

Next, we focused on the neuroendocrine response of rat pups to an acute opiate challenge following chronic postnatal treatment. This treatment paradigm does not span several other critical periods of neuroendocrine development, including the prenatal periods of opioid neuron formation and endocrine gland differentiation. However, it does allow us to determine whether tolerance develops during chronic administration in pups that demonstrate a neuroendocrine response. Rat pups were treated from postnatal day 1 until weaning (day 20) with methadone (5 mg/kg/day in two daily doses). This particular treatment regimen was chosen for several

reasons. First, the suckling period in the rat resembles, from the standpoint of neuroendocrine ontogeny, the last trimester of human gestation, during which most fetal growth and rapid ontogeny of neural control of hormone secretion occur. Therefore, disruption of neuroendocrine function would be most likely to produce the effects on offspring growth and development during this time period. Second, this treatment period does not involve maternal administration, and so is not complicated by effects on maternal physiology or by withdrawal at birth. The results of this study showed that postnatal administration of large doses of methadone had relatively little effect on basal levels of growth-regulating hormones. A transient decrease in serum thyroxine was observed during the early phase of treatment, and there was a transient decrease in CS as weaning was approached. Instead, endocrine effects of postnatal methadone administration were characterized by marked endocrine responses to each injection and by persistent and selective changes in neuroendocrine responsivity to opiate challenge (figure 7). For example, PRL and TSH responses to opioid challenge were abnormal on day 45, long after withdrawal, while GH and CS responses were normal (Kuhn and Bartolome 1984). In contrast, when adult rats are treated with a similar regimen, PRL responses to a challenge dose of opiate are not affected by even a more intensive dosage regimen, but CS responses remain abnormal for a long period of time after drug withdrawal (figures 8 and 9).

These results exemplify two opposite responses of the developing nervous system to insult. The persistent abnormality in PRL secretion typifies the effects expected following drug administration during development, i.e., the more prolonged disruption of a system following developmental exposure than after treatment during adulthood. However, in the same animals, CS secretion actually recovered faster in pups than it did when animals were treated chronically in adulthood: CS secretion actually was more perturbed when animals were treated in adulthood. This finding demonstrates the ability of the immature nervous system to repair damage through adaptations which are not possible in adult animals.

NEURAL PATHWAY MEDIATION OF OPIATE EFFECTS

At this point we made a critical decision to define more carefully the specific neural pathways mediating the PRL and CS responses which were different after chronic opioid treatment during development. We examined the effect of changing neurotransmitter mediation of opioid regulation of hormone secretion during ontogeny, and also on the ontogeny of different opioid receptor subtype systems. The results showed that the variable responses of developing animals to chronic morphine challenge might be explained by the changing neurotransmitter control of opiate-induced hormone secretion.

In adult rats, morphine-induced PRL secretion requires an intact serotonergic innervation of the hypothalamus from the dorsal raphe

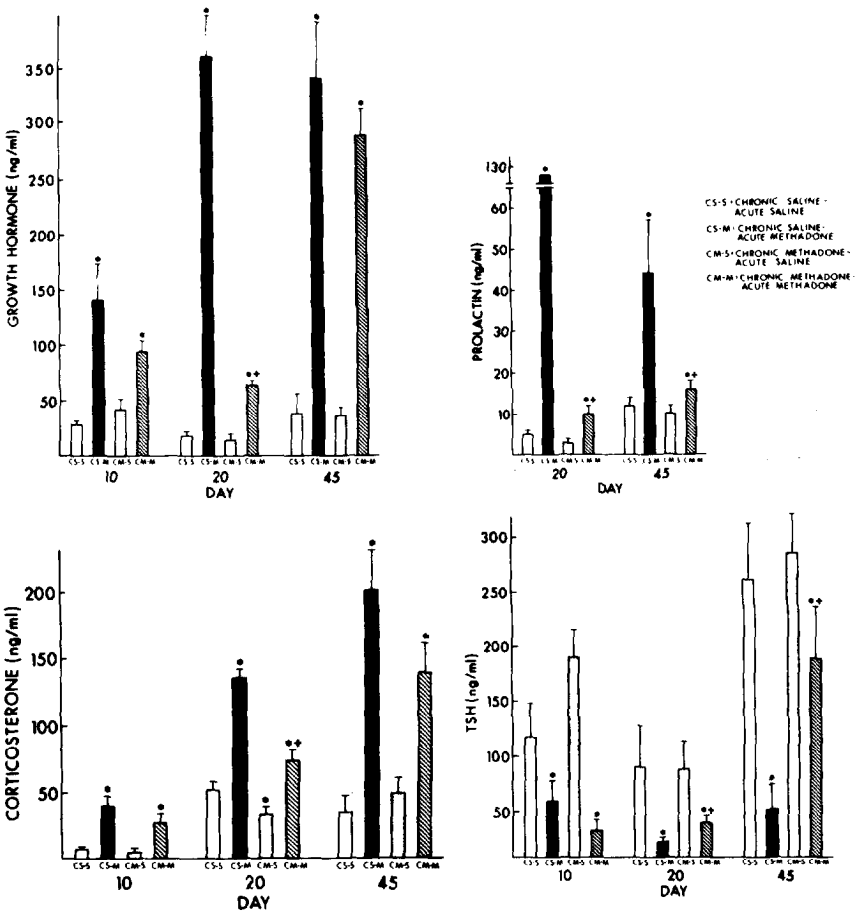

FIGURE 7. *Endocrine response to a challenge dose of methadone following chronic postnatal treatment*

KEY: C/S (clear bar) = chronic saline. acute saline; C/M (black bar) = chronic saline, acute methadone; M/S (stippled bar) = chronic methadone, acute saline; M/M (striped bar) = chronic methadone, acute methadone; * indicates significantly different from C/S control, p<.05 or better; + Indicates statistically dltterent from M/S control, p<.05 or better.

NOTE: Animals were treated with saline or methadone (5 mg/kg/day) for postnatal days 1 to 20 and given a challenge dose of methadone at the end of the treatment regimen (or 2 weeks later on day 45), killed, and their hormone levels were determined. Results are expressed as mean ± SEM. N=6-8 in each group.

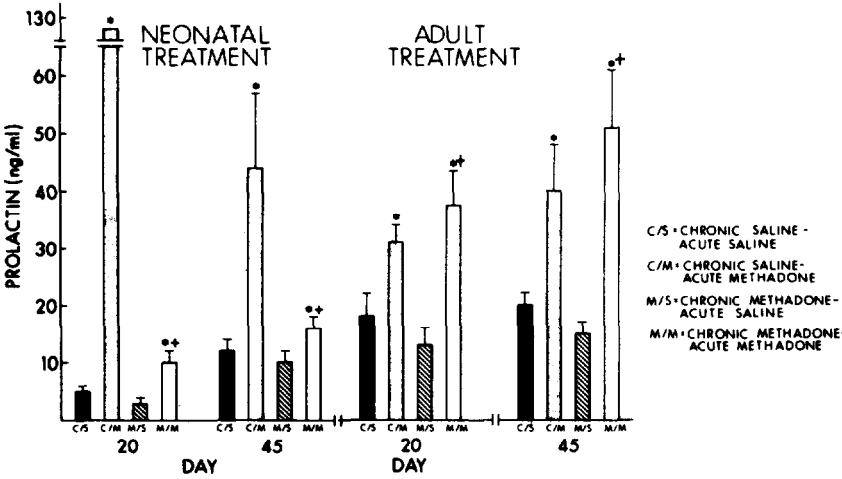

FIGURE 8. *Comparison of PRL response to acute challenge at the end (day 20) and 2 weeks after withdrawal (day 45) following postnatal or adult treatment with methadone (5 mg/kg/day)*

KEY: * Indicates statistically different from C/S control, p<.05 or batter;
+ Indicates statlstlcally different from M/S control, p<.05 or better.

NOTE: Animals were treated as described In figure 3. Results are expressed as mean ± SEM.

nucleus (Demarest and Moore 1981; Spampinato et al. 1979). However, other studies showed that functional serotonergic innervation of those hypothalamic neurons involved in regulating PRL secretion does not mature until the beginning of the third postnatal week (Becu and Libertun 1982; Cocchi et al. 1977). We considered the possibility that two different endogenous opioid systems were involved in regulation of PRL secretion: one a serotonin-independent system which matured early and the other a serotonin-dependent system which matured later and predominated over the earlier maturing system. To test this possibility, we investigated the ability of various pharmacologic treatments to impair morphine-induced PRL secretion in neonatal and adult rats. The results showed that blockade of serotonergic function by the antagonist cyproheptadine, or by chemical lesioning with 5,7 dihydroxytryptamine (DHT), eliminates morphine-induced PRL elevations in adult but not neonatal rats (figures 10 and 11). On the other hand, serotonergic mediation of opiate-induced analgesia can be demonstrated in neonatal rats. These findings suggest that specific opioid systems that mature early are particularly at risk for disruption by perinatal addiction, and, conversely, that

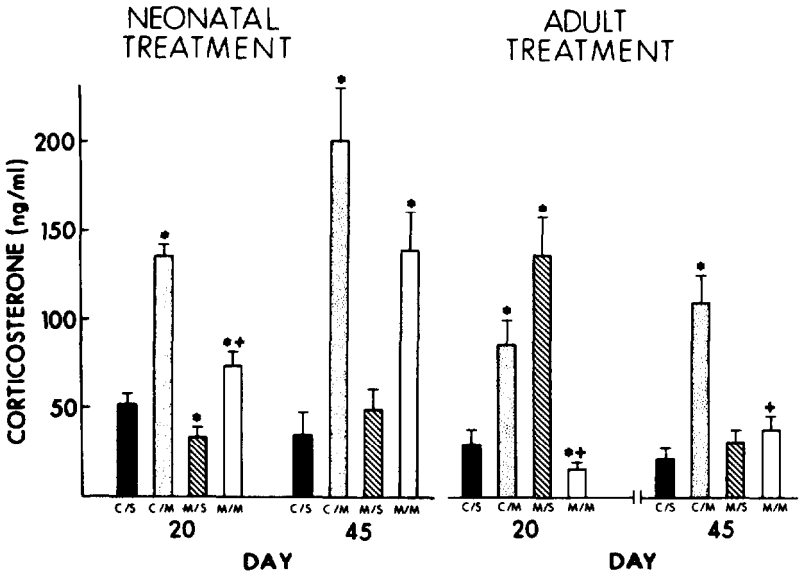

FIGURE 9. *Comparison of CS response to aaute challenge at the end (day 20) and 2 weeks after withdrawal (day 45) following postnatal or adult treatment with methadone*

NOTE: Animals were treated and results analyzed as described in figure 8.

systems like the serotonergic-opioid system regulating PRL secretion might be relatively resistant to treatments which precede establishment of functional transmission in the system.

ONTOGENY OF OPIATE RECEPTOR SUBTYPE-SPECIFIC RESPONSES AND CHANGING NEURAL PATHWAY MEDIATION OF OPIATE EFFECTS

The recent discovery of the presence of the three different endogenous opioid systems and their role in neuroendocrine ontogeny provides a specific neural mechanism to explain many acute and chronic opioid effects which were poorly understood, including the apparently disparate mechanisms involved in control of PRL secretion. The three different endogenous opioid systems are thought to have unique endocrine functions (Bero and Kuhn 1986; Spiegel et al. 1982; Koenig and Krulich 1984; Krulich et al. 1986a; Krulich et al. 1986b). The endorphin neurons with cell bodies in the arcuate nucleus are thought to utilize ß-endorphin as the major neurotransmitter and to mediate opioid effects on PRL, LH, and possibly ACTH through actions on μ receptors. Enkephalin neurons are widespread through the hypothalamus and are thought to act through δ (and possibly μ) opioid receptors, which have been implicated in control of LH and GH secretion. Finally, the endogenous opioid dynorphin is contained in cell bodies within the magnocellular nucleus and elsewhere in the hypothalamus, and is

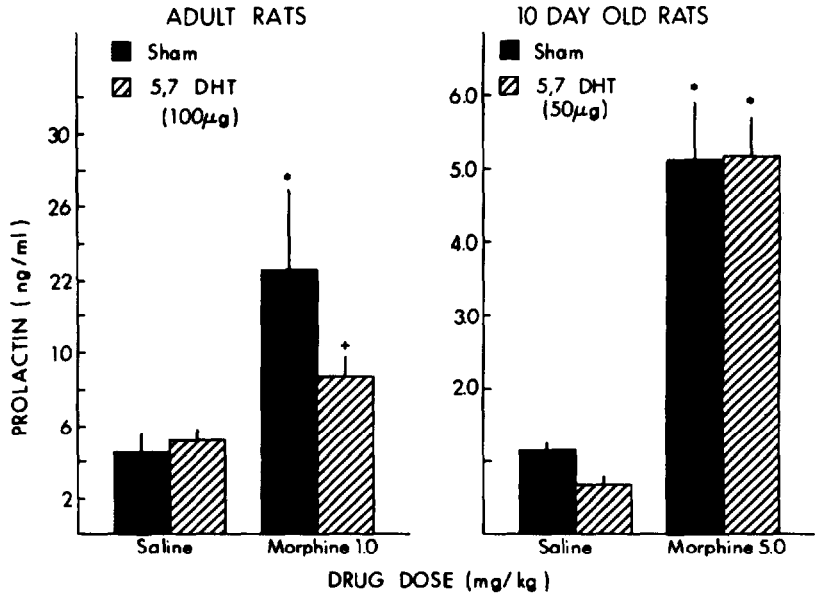

FIGURE 10. *Effect of 5,7 dihydroxytryptamine (5,7 DHT) treatment on PRL response to morphine in adult and neonatal rate*

KEY: * Indicates p<.05 or better relative to saline/saline control; + indicates p<.05 or bettor relative to morphine.

MOTE: Rats were pretreated with saline (solid bars) or 5.7 DHT (100 μg free base with desipramine. 20 mg/kg) (striped bars). Four days later, the animals were injected with morphine or saline 30 minutes before sacrifice. Results are expressed as mean ± SEM. N=6-8 In each group.

thought to modulate PRL, LH, and ACTH secretion through actions on κ ıreceptors. The differential ontogeny of these different opioid neuronal systems and their receptors provides a conceptual framework for understanding the specific developmental effects reported in a paper by Eisenberg (1985). Both μ and κ ıreceptor-mediated changes in PRL secretion are found in adult rats (Koenig and Krulich 1984). Therefore, we postulated that the serotonin-dependent and -independent systems may reflect actions of different opioid receptors with a differential ontogeny. To evaluate this possibility, we developed pharmacologic challenges for specific opioid receptor subtype systems and found that, in rat pups, as reported previously in adult rats (Koenig and Krulich 1984; Krulich et al. 1986a; Krulich et al. 1986b). PRL secretion

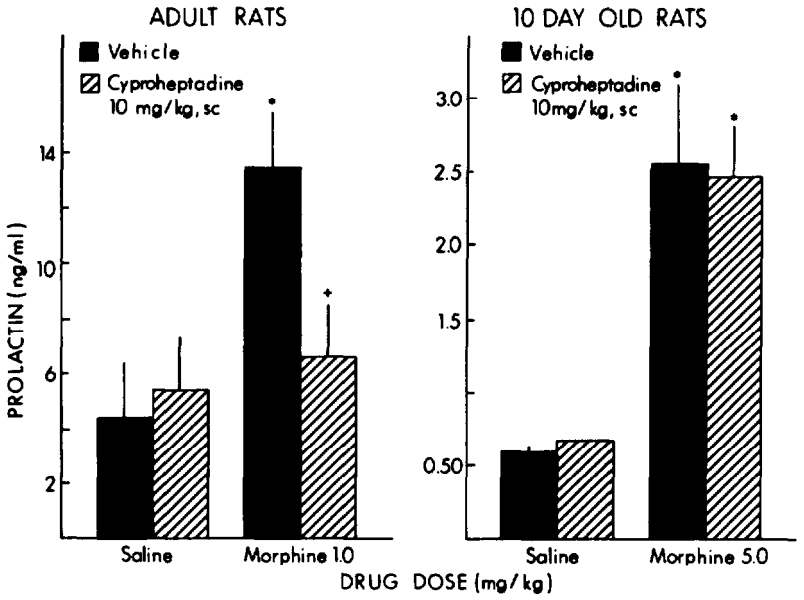

FIGURE 11. *Effect of cyproheptadine on PRL response to morphine in adult and neonatal rate*

KEY: * Indicates statistically different from saline-injected control;
+ indicates statistically different from morphine treatment, $p<.05$.

NOTE: Rats were pretreated with saline (solid bars) or cyproheptadine (10 mg/kg) (striped bars), followed 45 minutes later by saline or morphine. Animals were killed 30 minutes after the second injectIon. Results are expressed as mean ± SEM. N=6-8 In each group.

increases in response to small and probably receptor subtype-selective doses of the κ agonist (U50,488) and the μ agonist morphine (5 mg/kg).

By combining neurotransmitter antagonist treatments with these opioid receptor subtype-specific agonists, we have shown that the serotonin-dependent and -independent processes are mediated by opiate receptor subtypes which appear to have a differential ontogeny. In adults, PRL secretion stimulated by a μ receptor-selective dose of morphine is blocked by the serotonin antagonist cyproheptadine, while the PRL response to a small dose of the κ opioid agonist U50,488 is not affected (figure 12). In neonatal rats, PRL responses to κ agonists are larger and appear earlier than responses to μ agonists. Furthermore, responses to U50,488 and morphine are blocked by cyproheptadine in adult but not neonatal rats (figures 13 and 14). These findings then suggest an explanation for the tolerance development observed during chronic

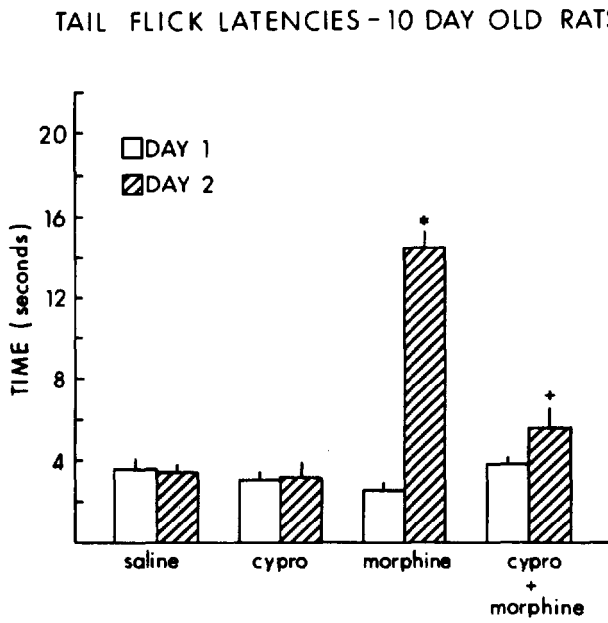

FIGURE 12. *Effect of cyproheptadine on tail flick latency in 10-day-old rate*

KEY: Open bars = tall flick latency on day 1; striped bars = tall flick latency on day 2; * indicates p<.05 relative to day 1; + indicates p<.05 relative to morphine on day 2.

NOTE: Vehicle or cyproheptadine was injected 45 minutes before saline or morphine. Analgesia was assessed 30 minutes later by the tall immersion method. The results are expressed as latency to withdraw tall from warm water. N=10 for each treatment group.

treatment of pups but not adults. The treatment paradigm in the neonatal experiment may have resulted in the selective stimulation of the κ receptors which were already functionally stimulating PRL secretion, while sparing the v-mediated, serotonin-dependent system which is not functional until the beginning of the third postnatal week. The tolerance evident during challenge on day 21 probably reflected, for the most part, the tolerance development at a single opioid receptor subtype (κ) that mediates the serotonin-independent mechanism for stimulating PRL secretion. The immediate question raised by these studies is whether the PRL results reflect a generalized phenomenon of more marked adaptation at κ than at μ opioid receptors, or simply the greater exposure of the early maturing κresponse.

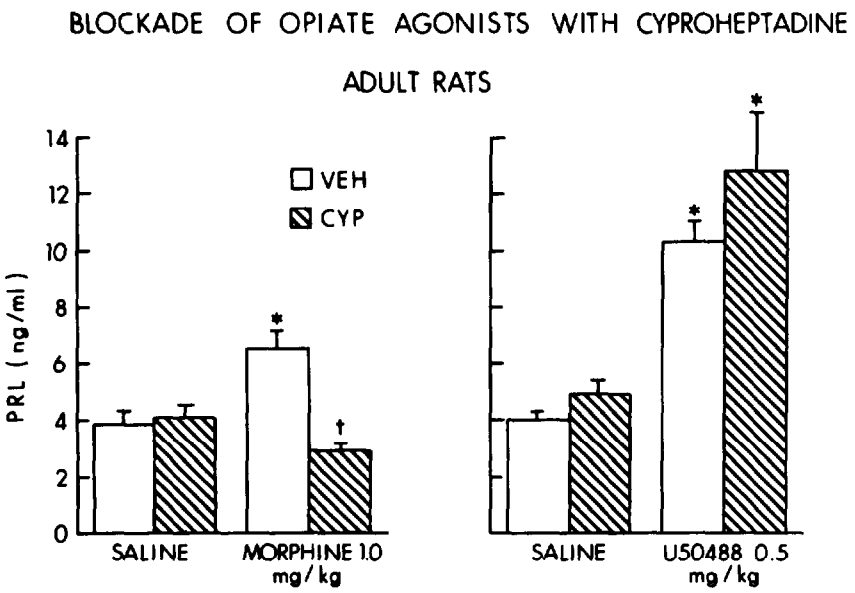

FIGURE 13. *Lack of inhibition by cyproheptadine of morphine or U50,488 effects on PRL secretion in adult rate*

KEY: * Indicates $p<.05$ relative to saline-injected control; † indicates $p<.05$ relative to morphine treatment.

NOTE: Animals were pretreated with vehicle (open bar) or cyproheptadine (10 mg/kg) (striped bar), followed by saline, morphine (1 mg/kg), or U50,488 (0.5 mg/kg). Animals were killed 30 minutes after the second injection, and PRL was determined. Results are expressed as mean ± SEM. N=6-8 In each group.

SUMMARY

In summary, we have shown that marked acute responses as well as persistent changes in hypothalamopituitary responsivity to opiate challenge result from perinatal opioid addiction. We have also shown that different endocrine systems and opioid receptor subtypes develop at different rates, and that the responses of these systems depend upon the relative timing of the treatment regimen and the functional development of the particular opioid system involved. It should be emphasized that these studies have investigated only a single developmental window. The additional critical question of how opioid neuron function is affected by treatment during the period of active neuronal differentiation has not yet been answered. However, these studies do demonstrate the utility of this neuroendocrine model in assessing opioid function following chronic treatment regimens. By using neuroendocrine function

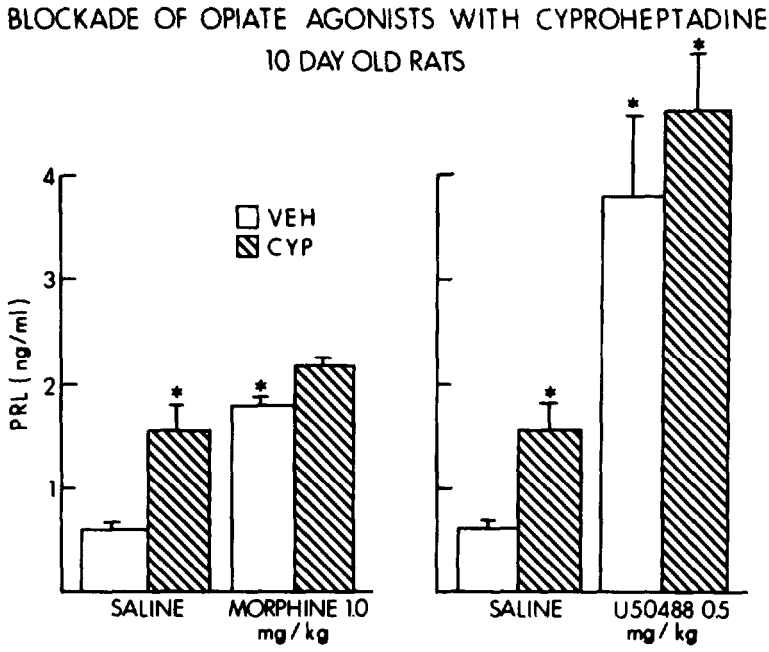

FIGURE 14. *Effect of cypoheptadine on morphine or U50,488 effects on PRL secretion in 10-day-old rats*

NOTE: Animals were treated and results expressed as described in figure 13.

as an end point, multiple systems can be studied simultaneously in the same animal. This has a particular advantage in studying the effects of chronic drug exposure on the developing nervous system, because hormone secretion is an easily quantifiable and early maturing functional index which can be used to identify vulnerable (and resistant) systems. Endogenous opioid systems appear to be particularly important in neuroendocrine regulation during the early phase of development, when other neural controls have not yet matured. Our preliminary results suggest that specific opioid systems that mature early may be especially important in the specific neuroendocrine effects of perinatal opiate addiction.

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Cellular Mechanisms of Noradrenergic Enhancement of Long-Term Synaptic Potentiation in Hippocampus

Daniel Johnston, William F. Hopkins, and Richard Gray

INTRODUCTION

The action of norepinephrine (NE) in the functioning of the central nervous system (CNS) is not known. However, the diffuse projections of noradrenergic fibers throughout the cerebral cortex (Moore and Bloom 1979) have led to the belief that this system plays some neuromodulatory role (Kety 1970; Woodward et al. 1979; Foote et al. 1983). Evidence has been presented to suggest that NE is involved in the plasticity of the developing nervous system (Pettigrew and Kasamatsu 1978; Bear and Singer 1986), in controlling the behavioral states of an animal (Kety 1970; Aston-Jones and Bloom 1981a; Aston-Jones and Bloom 1981b), and in learning and memory (Crow 1968; Ogren et al. 1980; Mason 1981). The control of behavioral states may be particularly relevant to this symposium, because if drugs of abuse have receptors on locus coeruleus neurons or their axon terminals, it would be expected that they would modify the output of the noradrenergic system and possibly the behavioral state of the organism.

NE-containing fibers have also been shown to innervate the hippocampus diffusely. The areas most densely innervated are the hilar region of the dentate gyrus and stratum lucidum of CA3 (Moore and Bloom 1979). Several years ago, we began an investigation of the noradrenergic effects on mossy fiber synaptic transmission. Mossy fibers are the axons from dentate granule cells, and they form synapses onto CA3 pyramidal cells. These synapses are excitatory and have many unique characteristics. For example, the synaptic endings are unusually large--approximately 5 μm in diameter (Amaral 1979). They contain a high concentration of zinc (Haug 1967) (the function of which is unknown), and they terminate on the proximal portion of the apical dendrites of the pyramidal neurons (Johnston and Brown 1983). This latter characteristic has enabled the use of voltage-clamp techniques for the study of the biophysical properties of this synapse (Brown and Johnston 1983; Johnston and Brown 1983).

The close anatomical location of the mossy fiber synapses to the somata of the CA3 neurons was shown to be correlated with a very small electrotonic distance (Johnston and Brown 1983). Thus, a voltage clamp applied to the somata of the CA3 neurons can provide a reasonable voltage clamp of the mossy fiber subsynaptic membrane. Because of this unique situation, Brown and Johnston (1983) were able to determine many of the biophysical parameters of this synapse, including its conductance, reversal potential, and kinetics. The mossy fiber synapse thus serves as one of the best characterized (at a biophysical level) excitatory synapses in the mammalian CNS.

Long-term synaptic potentiation (LTP) was first described by Bliss and Lomo (1973). They showed that brief tetanic stimuli given to excitatory synaptic pathways resulted in a long-term enhancement of synaptic efficacy. Considerable interest has been focused on the mechanisms and properties of LTP at synapses in the hippocampus and elsewhere (Bliss and Dolphin 1982: Swanson et al. 1982). Because of the unusual properties of the mossy fiber synapses. we were oarticularlv interested in the features of LTP at these-synapses. In parallel with our studies of LTP, we developed a new preparation in which patch-clamp techniques could be applied to adult cortical neurons (Gray and Johnston 1985). These methods enabled us to investigate possible membrane mechanisms associated with LTP.

In this review, we will describe studies in which we found that NE, through ß-adrenergic receptors, enhances the magnitude, duration, and probability of induction of LTP at mossy fiber synapses (Hopkins and Johnston 1984; Hopkins and Johnston, in press). Using patch-clamp techniques on hippocampal granule cells, we also found that ß-adrenergic receptors enhance the activity of voltage-dependent calcium channels (Gray and Johnston, in press). The results from these two areas of investigation have led to our current hypothesis that NE enhances LTP through increased calcium influx into presynaptic elements or postsynaptic elements, or both.

METHODS

Methods for extra- and intracellular recordings from rat hippocampal slices have been described in detail elsewhere (Brown and Johnston 1983; Johnston and Brown 1983; Hopkins and Johnston 1984). All drugs were bath-applied, and ascorbate (20 µM) was routinely added to both control and catecholamine-containing solutions. For extracellular recordings, micropipettes of 1 to 10 MΩ were filled with 2 M sodium chloride and placed under visual guidance in stratum pyramidale and stratum lucidum of the CA3 subfield. Mossy fibers were stimulated with stainless steel bipolar electrodes positioned in the hilus close to stratum granulosum in the dentate gyrus (see figure 1). Field potentials were evoked with brief, low-intensity (0.05 msec, 5-100 µA), constant-current pulses and were averaged on-line by a laboratory computer. Low-intensity stimuli were used to reduce the probability of ohserving

epileptiform activity, which is sometimes evoked when multiple high-frequency trains are used.

The techniques for patch-clamping acutely exposed neurons from adult hfppocampal slices have also been described elsewhere (Gray and Johnston 1985; Gray and Johnston, submitted for publication). Slices were treated with proteolytic enzymes for 15 to 20 minutes and then shaken gently to split the slices along the cell body regions, exposing the neural somata. The exposed neurons were then patch-clamped in the whole-cell or cell-attached configuration, using conventional techniques (Hamill et al. 1981). The compositions of the pipette and bath solutions were chosen to block all potassium and sodium currents, and drugs were applied by pressure-ejection through a single- or double-barrelled puffer pipette placed near the soma of the clamped cell.

RESULTS

The initial set of experiments was designed to determine whether bath-applied NE affected synaptic efficacy. One method of examining this question is to compare the input-output characteristics and responses in normal saline with those obtained in the presence of NE. The input-output curve in our experiments represents the amplitude of the population excitatory postsynaptic potential (pEPSP) as a function of the stimulus current intensity used to activate the mossy fibers. This population response is measured extracellularly and results from the synchronous activation of a number of mossy fiber synapses. Figure 2 illustrates the findings of a typical experiment. With the mossy fibers stimulated at a frequency of 0.2 Hz, the addition of 10 μM NE to the bath had little or no effect on the amplitude of the pEPSP. In other experiments, NE sometimes produced a small rightward shift in the input-output curve. However, we never observed a leftward shift. A leftward shift would be indicative of an increase in synaptic efficacy; a rightward shift would represent a decrease in synaptic efficacy. We concluded from these results that, under conditions of low-frequency stimulation, NE did not augment the efficacy of mossy fiber synapses.

When NE was present during high-frequency stimulation, the results were auite different. Fiaure 3 shows the results of one of the types'of experiments used-to test for NE's effects on LTP. Slices were chosen in which we could obtain a control episode of LTP whose amplitude decreased over approximately 60 minutes. NE was then added to the bath, and another high-frequency stimulus train was given to induce a second episode of LTP. With NE present in the bath only immediately before and during the high-frequency train, the magnitude and duration of LTP were increased. The third episode of LTP was in normal saline and yielded magnitude and duration values similar to those of control. In other

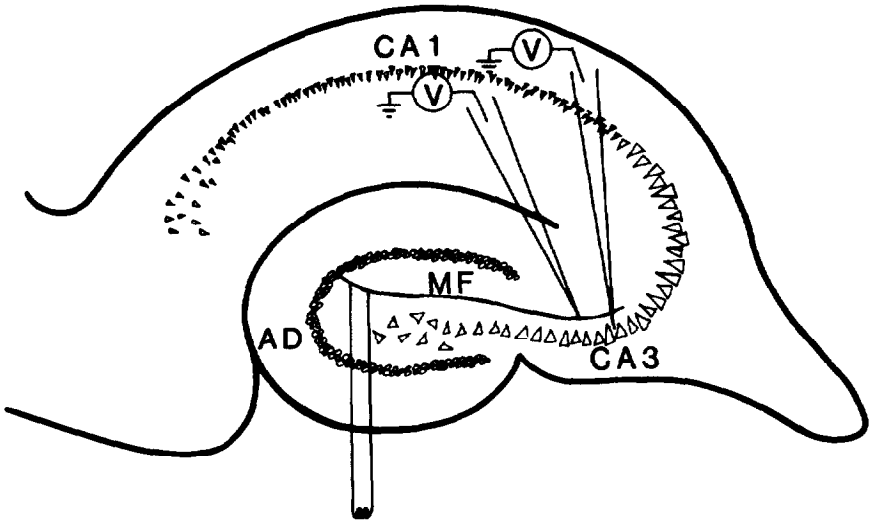

FIGURE 1. *Schematic diagram of the hippocampal slice preparation*

KEY: MF=mossy fibers, AD=area dentata. V=voltage recording amplifier.

NOTE: A bipolar stimulating electrode was placed adjacent to the hilar side of stratum granulosum of the dentate gyrus, and recording electrodes were positioned In stratum pyramidale and stratum lucidum of CA3 to record the extracellular population spike and EPSP, respectively.

SOURCE: Hopkins and Johnston (submitted).

experiments (data not shown), NE also increased the probability of inducing LTP when low-intensity stimulus trains were given to the mossy fibers. A number of pharmacological experiments were performed, the results of which suggested that the NE enhancement of LTP is probably mediated by ß-adrenergic receptors through an increase in cyclic adenosine monophosphate (cAMP) (Hopkins and Johnston 1984).

These results led to the hypothesis that NE increases the magnitude, duration, and probability of induction of LTP through ß-adrenergic receptors and cAMP. The effects of NE on mossy fiber synapses also appeared to be dependent on the frequency of stimulation. At low stimulus frequencies, NE either had no effect or tended to depress synaptic transmission, whereas when NE was present during a high-frequency stimulus train, there was an enhancement of synaptic efficacy.

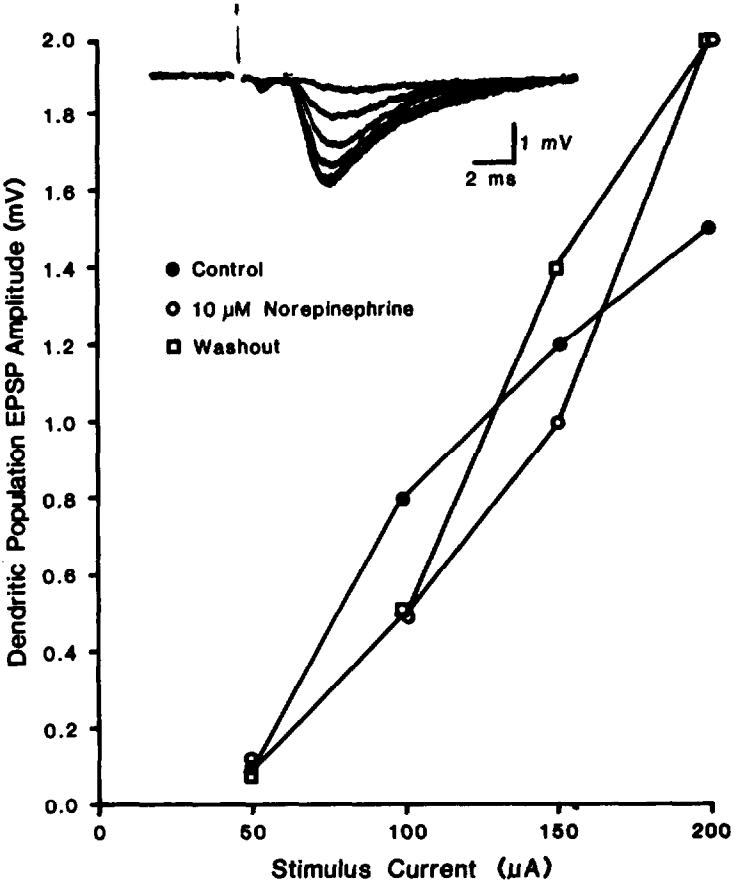

FIGURE 2. *Input-output curves for the pEPSP vs. stimulus current intensity before, during, and after bath application of 10 µM NE*

NOTE: The inset depicts examples of pEPSPs evoked by a series of different stimulus current intensities.

SOURCE: Hopkins and Johnston (submitted).

The obvious question that arises from these investigations is, By what cellular mechanism(s) does NE modulate LTP? To address this question, we felt it necessary to develop a new preparation In which patch-clamp techniques could be applied to adult cortical

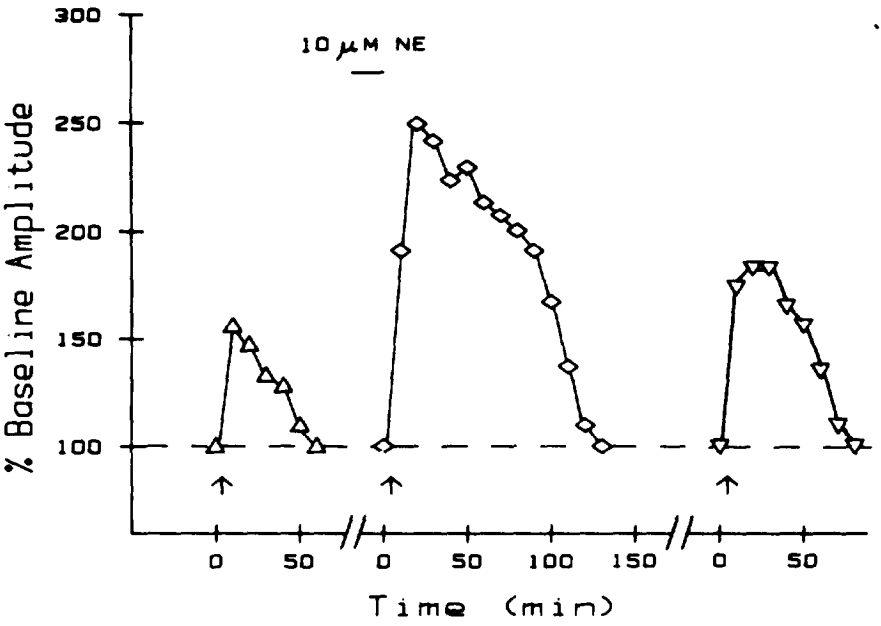

FIGURE 3. *Effects of brief bath apptication of NE (10 μM) on LTP*

NOTE: In this experiment, NE was present 20 minutes before and during tetanic stimulation but was washed out immediately after the conditioning train. Arrows indicate time of conditioning train (100 Hz, 2 sec, 50 μA) for each episode.

SOURCE: Hopkins and Johnston (submitted).

neurons. This preparation is shown schematically in figure 4. As described earlier, slices from adult guinea pigs were treated with proteolytic enzymes and agitated gently until the slices split apart along the cell body region, exposing pyramidal cells and granule cells at the edge of the slice. We have used this preparation to study ligand- and voltage-gated ion channels from cell-attached, excised-patch, or whole-cell configurations (Gra and Johnston 1985; Gray and Johnston, submitted for publication).

Although the mechanisms underlying LTP in hippocampus (or elsewhere) are unknown, one finding that appears to be consistent in many laboratories is that LTP is critically sensitive to calcium (Dunwiddie and Lynch 1979; Turner et al. 1982); thus, calcium influx into at least the postsynaptic neuron is a prerequisite for LTP (Lynch et al. 1983). A plausible working hypothesis directing our initial experiments was that the modulation of LTP by NE could

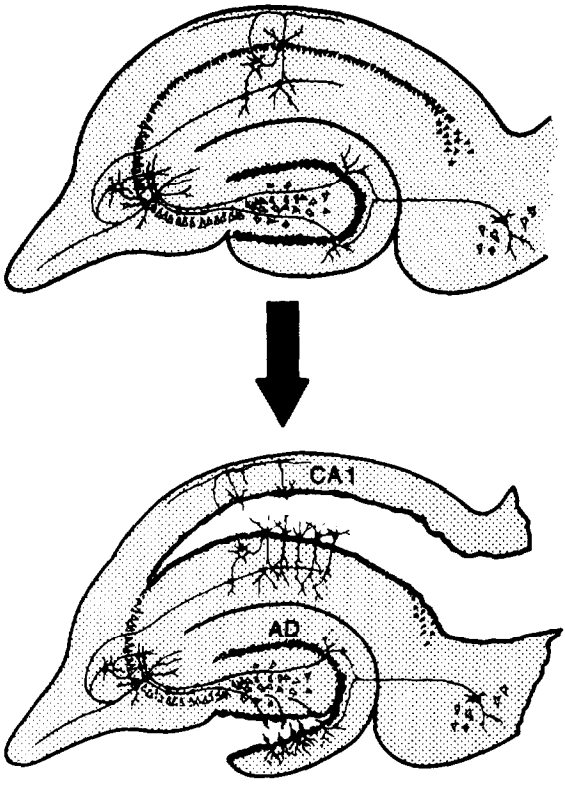

FIGURE 4. *Schematic drawing of a hippocampal slice before (above) and after (below) it was split along cell body regions to expose pyramidal neurons and dentate granule cells*

NOTE: The slice was split along the major cell body layers (pyramidal CA1 and area dentata (AD)), exposing the somata and proximal processes.

SOURCE: Gray and Johnston (1985). Reprinted by permission from J Neurophysiol 54:134-142, Copyright 1985, the American Physiological Society.

be mediated through a modulation of calcium currents, It is difficult to obtain voltage-clamp recordings of calcium currents using traditional microelectrode techniques because calcium currents are relatively small and hard to measure in the presence of potassium currents. Potassium currents are usually much larger than calcium currents, and it is difficult to block them completely by drugs applied to the external bath. Using a whole-cell recording technique, however, the cell can be internally dialyzed with ionic substitutions and drugs that block potassium channels, allowing a much better separation of calcium currents from

potassium currents. In our experiments, we replaced internal potassium with cesium ions, which are impermeable through potassium channels, and added two drugs (tetraethylammnonium (TEA) and 3,4,diaminopyridine (DAP)) known to block several different potassium channels to both the inside and outside solutions. Tetrodotoxin (TTX) was also added to the bath to block voltage-dependent sodium conductances. These manipulations allowed us to record relatively pure voltage-dependent calcium currents without contamination from currents flowing through potassium or sodium channels.

Figure 5A shows results of an experiment in which we measured the peak amplitude of the calcium current before and after the application of NE to the external surface of a hippocampal granule cell. A small, but significant, increase in the peak inward

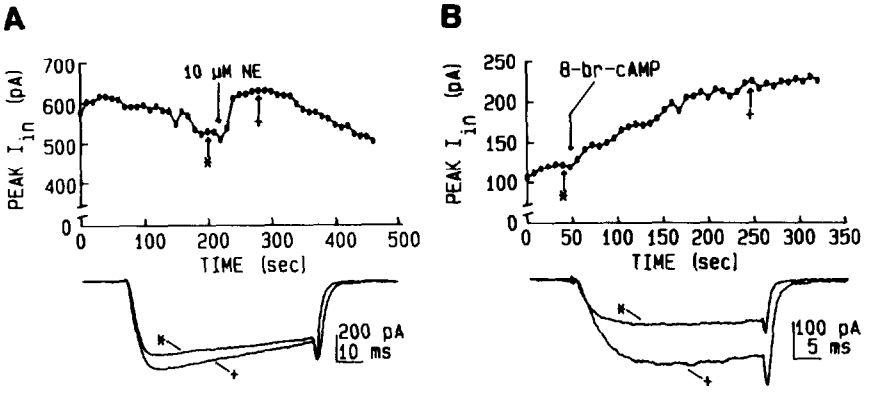

FIGURE 5. *Application of norepinephrine or 8-bromo-cAMP increased the peak inward calcium current*

NOTE: (A) Plot of peak Inward current vs. time after formation of whole-cell clamp. Above. a 500-ms puff of 10 µM NE was applied at the time indicated by the downward arrow. Sample traces before (*) and after (+) drug application are shown below. The charge carrier in this experiment was calcium. Holding potential, -60 mV; command potential, 0 mV. (B) Identical experiment, except that a 500-ms puff of 8-bromo-cAMP was applied at the arrow, above. Sample traces before (*) and after (+) drug application are shown below.

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calcium current occurred with 10 µM NE. In other experiments (data not shown), we found that the ß-adrenergic receptor isoproterenol had effects similar to those with NE. leading to the hypothesis that the enhanced calcium current is mediated through activation of ß-adrenergic receptors. Because the activation of ß-adrenergic receptors has been shown to increase the levels of

cAMP in hippocampus (Bloom 1975; Segal et al. 1981), we investigated whether cAMP would mimic the action of NE. Figure 5B shows the results of an experiment using the membrane-permeable analog 8-bromo-cAMP. We found that it also produced a very significant enhancement of the peak calcium current.

To explore further the enhancement of voltage-dependent calcium currents by cAMP, we recorded the activity of single calcium channels from cell-attached patches. Figure 6 illustrates the results of two experiments. Figure 6A is from an experiment in which calcium channel activity was measured in response to step depolarizations during control (1) and after application of 8-bromo-cAMP to the surface membrane of the cell (2). We measured the fractional open time by determining the length of time during a command that the current exceeded a preset threshold level. The graph on the upper right (3) shows that when 8-bromo-cAMP was applied, the mean fractional open time increased from about 0.2 to about 0.3. Figure 6B is from another experiment in which a patch contained multiple calcium channels. A similar experiment was performed in which we measured channel activity during commands and averaged all of the commands under control conditions (1) and after application of 8-bromo-cAMP (2). The ensemble average currents are shown in the lower right panel (3) before and after the addition of 8-bromo-cAMP. A small, but significant, increase in the average channel activity, similar to the results obtained with the whole-cell recording techniques, was produced by 8-bromo-cAMP.

DISCUSSION

In summary, these results indicate that NE enhances the magnitude, duration, and probability of induction of LTP at mossy fiber excitatory synapses. Furthermore, we found that NE increased voltage-dependent calcium conductance. The obvious conclusion from these investigations is that the augmented LTP results from increased calcium influx. The action of NE in increasing calcium currents is in contrast to its effects on neurons from peripheral ganglia, in which it appears to decrease calcium currents (Dunlap and Fischbach 1978; Dunlap and Fischbach 1981). However, NE has been shown to increase calcium conductance in ventricular heart cells through ß-adrenergic receptors (Cachelin et al. 1983; Reuter 1983; Bean et al. 1984). The increase in voltage-dependent calcium current, which we observed in hippocampal neurons, could lead to significant changes in synaptic transmission if NE enhancement of the calcium current occurred presynaptically. Moreover, results from other laboratories strongly suggest that calcium influx into the postsynaptic neuron is a requirement for the mechanisms of LTP (Lynch et al. 1983). If NE increased calcium currents postsynaptically, an enhanced LTP might result. It is not known whether LTP is modulated by NE elsewhere in the brain; however, if the NE enhancement of calcium conductance mechanisms occurs elsewhere, NE could be expected to modulate numerous neuronal processes that are linked to calcium influx into pre- or postsynaptic elements.

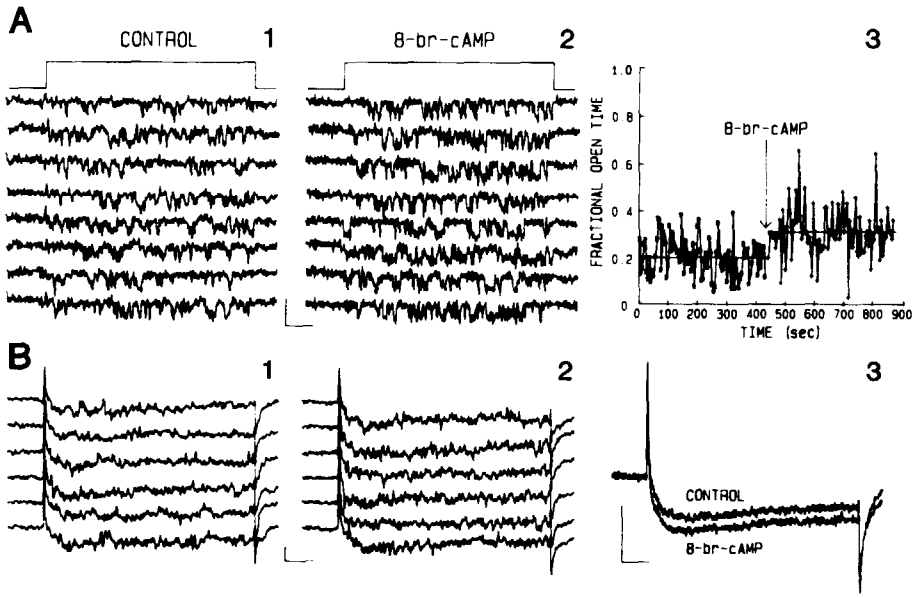

FIGURE 6. *Application of 8-bromo-cAMP increased the activity of calcium channels*

NOTE: Single-channel activity from cell-attached patches on granule cells. Patch pipettes contained 96 mM $BaCl_2$; 100 µM 3,4-DAP; 1 µM TTX; and 10 mM HEPES. The pH was adjusted to 7.35 with TEA-OH. The exposed cells were bathed In normal extracellular saline. (A1) Consecutive traces of channel activity in control saline In response to step commands from -60 to +70 mV, with respect to the unknown resting potential. (A2) Channel activity in the same patch 10 s after a 500-ms pressure pulse was applied to a puffer pipette that was located near the cell body and contained normal extracellular saline plus 5 mM 8-bromo-cAMP. (A3) Plot of the fraction of time during the command when the current level was more negative than a threshold level (-0.25 pA) before and after application of 8-bromo-cAMP. The threshold was set at -3 times the standard deviation of the baseline noise calculated from traces In which no channel activity was visible. (B1) Control channel activity In a different patch (different granule cell from that in A) in response to a command from -40 to +60 mV with respect to the unknown resting potential. The patch contained many channels. (B2) Channel activity in the same patch after a 500-ms application of 5 mM 8-bromo-cAMP. (B3) Ensemble averages of 56 traces before and 56 traces after drug application. All current traces shown were filtered at 2 kHz (-3 dB, Bessel response). All calibration bars are 1 pA x 10 ms.

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Neuronal Adaptation in the Hippocampus Induced by Long-Term Ethanol Exposure

Don W. Walker and Bruce E. Hunter

INTRODUCTION

Chronic and excessive use of alcohol is widely acknowledged to result in brain damage and associated functional deficits. The evidence supporting this statement has emerged from research investigating cognitive, morphological, electrophysiological, and biochemical abnormalities in chronic alcoholic patients (Begleiter and Platz 1972; Cala and Mastaglia 1981; Freund 1973; Parsons 1977; Porjesz and Begleiter 1981; Ron 1977; Thomson and Ron 1982). Ethanol is a unique drug in that it contains calories (7 kcal/g) which can replace nutrient calories, resulting in the potential for malnutrition. In fact, alcoholic brain damage has traditionally been attributed to malnutrition, especially thiamine deficiency, rather than to the neurotoxic effects of ethanol per se (Dreyfus 1974; Victor et al. 1971). It seems likely, however, that ethanol can directly exert neurotoxic effects because brain damage and cognitive impairment are observed in alcoholics with no history or evidence of malnutrition, head trauma, or exposure to other toxic agents or drugs (Brewer and Perrett 1971; Epstein et al. 1977; Freund 1973; Lynch 1960; Smith et al. 1973). Although the relative contribution of ethanol exposure per se is obviously difficult to address experimentally in human studies, controlled animal experiments from a number of laboratories have provided strong evidence for a direct neurotoxic effect of ethanol, though the mechanism of this toxicity is unknown (cf. Walker et al. 1981; Walker et al. 1982).

For the purposes of this discussion, chronic ethanol treatment (CET) will be considered as the duration of continuous ethanol exposure likely to result in lasting (or permanent) structural and functional alteration of neurons, which, in our experience, requires a minimum of 3 months of treatment. Ethanol exposure for only a few days, by contrast, while inducing tolerance and/or physical dependence, produces a disturbance of neuronal function thought to last for only a few days. Although neuronal damage associated with chronic or prolonged ethanol exposure can occur at levels of ethanol intake insufficient to produce evidence of

physical dependence (Walker and Freund 1971; Walker et al. 1980b), it is obvious that both ethanol neurotoxicity and the development of physical dependence could occur simultaneously. In studying the neuronal alterations associated with prolonged ethanol treatment, it is thus difficult to separate the neurotoxic changes from the more transient changes associated with the ethanol withdrawal reaction unless a sufficiently long posttreatment ethanol-free period is given prior to data collection, or data are collected at several time points following the ethanol treatment.

Collectively, research from our laboratory and others has provided convincing evidence of ethanol neurotoxicity despite more than adequate nutrient intake:

(1) Behavioral. In rodents, CET produces deficient learning of several behavioral tasks including shuttle box avoidance (Freund 1979; Freund and Walker 1971; Irle and Markowitsch 1983; Sotzing and Brown 1976; Triet et al. 1980; Walker and Freund 1971), temporal discrimination (Denoble and Begleiter 1979; MacDonall and Marcucella 1978; Smith et al. 1979; Walker and Freund 1973), complex maze learning (Bond and DiGiusto 1976; Fehr et al. 1976), temporal alternation (Walker and Hunter 1978), and spontaneous alternation (Beracochea and Jaffard 1985; Walker et al. 1981).

(2) Anatomical. CET induces morphological abnormalities in the rodent hippocampus and cerebellum, including neuronal degeneration and loss (Irle and Markowitsch 1983; Kunz et al. 1976; Lescaudron and Verna 1985; Phillips and Cragg 1983; Roulet et al. 1985; Tavares and Paula-Barbosa 1982; Walker et al. 1980a; Walker et al. 1980b; Walker et al. 1981; Walker et al. 1982) and altered dendritic structure and spine density (Goldstein et al. 1983; King et al. 1985b; Kunz et al. 1976; McMullen et al. 1984; Pentney 1982; Popova 1983; Riley and Walker 1978; Tavares et al. 1983a; Tavares et al. 1983b; Walker et al. 1981).

(3) Neurophysiological. CET induces complex changes in the function of hippocampal neurons, including reduced intrinsic inhibition (Abraham et al. 1981; Durand and Carlen 1984a; Rogers and Hunter 1985), deficient development of long-term potentiation in area CA1 (Durand and Carlen 1984b) and the dentate gyrus (Abraham et al. 1984), and altered distribution of afferent synaptic connections in CA1 and the dentate gyrus (Abraham and Hunter 1982; Abraham et al. 1982).

Our recent research has focused on the use of the rat hippocampus as a model system to investigate the dynamic characteristics, selectivity, and potential mechanisms of the effect of CET on neuronal structure, connectivity, and function. The hippocampus was chosen for a number of reasons, including: (1) The short-term memory deficit observed following chronic alcohol consumption in the rat (Beracochea and Jaffard 1985; Walker and Hunter 1978) is strikingly similar to that seen in chronic alcoholic patients

(Butters and Cermak 1975), patients with hippocampal lesions (Milner 1959; Milner et al. 1968). and rats with hippocampal lesions (Walker and Means 1973). (2) The vast available information on the normal anatomy, electrophysiology, and pharmacology of the hippocampus provides a significant data base. (3) The relatively simple and highly laminated structure of the hippocampus facilitates quantitative morphological and physiological measurement, thereby enhancing detection of possibly subtle alterations induced by CET. (4) The hippocampus is apparently sensitive to CET, since a number of laboratories have reported structural and physiological alterations in the hippocampus after CET.

Because the hippocampus is the brain structure serving as the model system for the work discussed below, a brief description of the basic anatomical features that facilitate quantitative analysis will be provided.

Figure 1 is a schematic transverse section illustrating the organization, major divisions, and principal cell types of the hippocampus. The hippocampal complex is divided into two regions: the hippocampus (Ammon's horn or cornu ammonis (CA)) and the dentate gyrus (DG). Figure 1 shows the relationship of these regions in cross section. The intrinsic synaptic organization of the hippocampus is marked by several salient characteristics. (1) There is an intrinsic trisynaptic series of excitatory connections beginning with a projection from the entorhinal cortex to the DG, the DG to CA3, and finally CA3 to CA1 (Gottlieb and Cowan 1973; Guthrie and Elliott 1980; Hjorth-Simonsen 1973; Hunter et al. 1986; Laurberg 1979; Swanson et al. 1978). (2) There is a laminar arrangement of morphological elements in the hippocampus. The CA and DG are basically trilayered; in each, a relatively tightly packed principal cell layer is bordered on either side by layered neuropil which is cell poor (figure 1). (3) The specific afferent terminations of the hippocampus are also laminated, so that each class terminates upon relatively specific portions of the dendrites of pyramidal (CA) and granule (DG) cells. (4) The remarkable lamellar organization of the hippocampus is such that a slice transverse to the longitudinal axis of the hippocampus only 300 to 500 μm thick preserves the basic trisynaptic series of intrinsic connections.

Previous research has shown that CET produces a 10- to 30-percent loss of both pyramidal and dentate gyrus granule cells in the rodent hippocampus, depending on the duration of exposure and other variables (Irle and Markowitsch 1983; Kunz et al. 1976; Lescaudron and Verna 1985; Phillips and Cragg 1983; Walker et al. 1980b; Walker et al. 1981). Because of the intrinsic and commissural monosynaptic connections of the hippocampus (Fricke and Cowan 1978; Laurberg 1979), this neuronal loss necessarily results in partial deafferentation of surviving hippocampal and dentate gyrus neurons. Furthermore, because deafferentation in the hippocampus is known to stimulate synaptic reorganization, including sprouting of remaining afferents (Cotman and Nadler 1978), CET is likely to

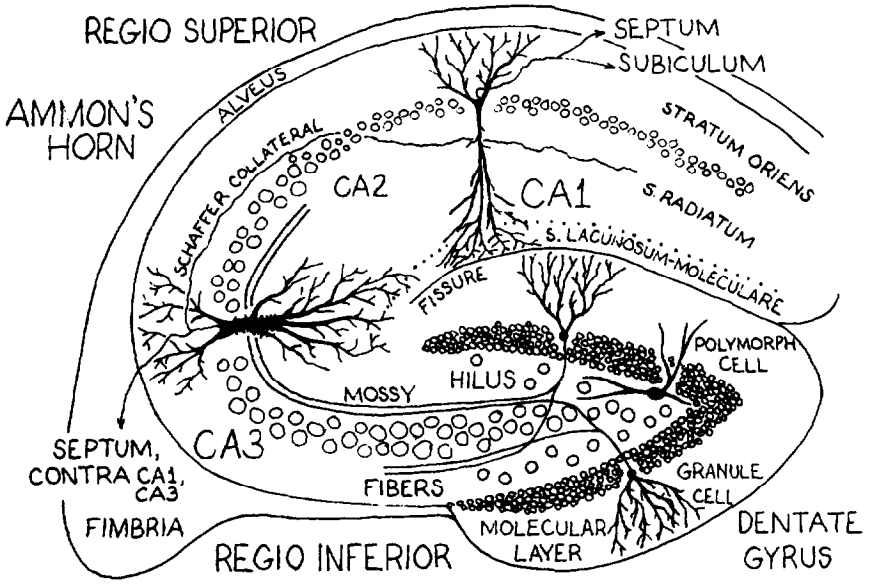

FIGURE 1. *A schematic transverse section through the hippocampal formation illustrating the divisions, organization, and principal cell types*

produce a complex sequence of changes including destructive cell loss, deafferentation, and synaptic reorganization. The surviving neurons will likely obtain some new targets for their projections and/or receive additional afferents to replace those which were lost. It is likely that destructive and compensatory changes occur simultaneously during CET and that further reorganizational or regenerative changes may occur following CET. As a result, some quantitative Golgi studies have found decreased dendritic branching and decreased density of dendritic spines on hippocampal cells surviving CET (Goldstein et al. 1983; McMullen et al. 1984; Riley and Walker 1978), whereas others report normal (Lee et al. 1981; Lescaudron and Verna 1985) or increased (Kunz et al. 1976) dendritic spine density on surviving CA1 pyramidal cells. The variability in the reported effects of CET on dendritic spine density in the hippocampus is not surprising, because it is apparent that each of the above results could be expected if destructive and compensatory changes are occurring simultaneously during CET. In other words, some cells destined to die may exhibit dendritic dying back and loss of synapses preceding death. Other cells not

damaged by CET may become hyperinnervated by gaining afferents that have lost contacts with dead or dying cells.

With these considerations in mind, we have concentrated our recent efforts on characterizing the CA1 pyramidal cells and DG granule cells that survive CET. The following questions have been pursued:

(1) Are there changes in the spatial extent, pattern, and/or density of specific afferents of surviving hippocampal neurons following CET?

(2) Are there changes in the synaptic function of surviving hippocampal neurons following CET?

(3) Do hippocampal neurons damaged or altered by CET recover during abstinence?

(4) What is the effect of CET on the capacity of surviving hippocampal neurons for reactive synaptogenesis?

In order to study these questions, we have standardized our CET parameters both to facilitate comparison across experiments and to allow comparison with our previous work. Ethanol was administered to mature, male Long-Evans rats via a nutritionally controlled liquid diet (Walker and Freund 1971) for 20 weeks (group E). Control groups (group S) were individually pair-fed an identical diet with sucrose substituted isocalorically for ethanol or were given free access to pelleted laboratory food. Except where noted (e.g., recovery series), an ethanol-free period of 8 weeks was imposed before experimentation began in order to control for the possible residual effects of ethanol withdrawal. The average daily ethanol consumption under these conditions is 12 to 15 g/kg, resulting in a blood-ethanol concentration of 100 to 200 mg/kg. All morphological data are collected in coded material, and all electrophysiological experiments are performed on coded animals. The codes are not broken until all data collection is completed.

SYNAPTIC CONNECTIVITY AND FUNCTION OF HIPPOCANPAL NEURONS SURVIVING CET

In order to fully characterize alterations in the afferent synaptic distribution and/or synaptic function of CA1 pyramidal cells and DG granule cells that survive prolonged ethanol exposure, we have combined the use of both electrophysiological and anatomical techniques.

Electrophysiological Analysis of Afferent Synaptic Distribution in the Hippocampal Formation After CET

Electrical stimulation in the stratum radiatum (SR) of CA1 or in the angular bundle (fibers from the entorhinal cortex) produces positive-negative dipole fields in CA1 and the dentate gyros (DG), respectively. In the synaptic field, one sees a negative field

potential called the extracellular excitatory postsynaptic potential (EPSP), which reflects summated excitatory synaptic activity (Lomo 1971). As the recording electrode is moved toward the cell layer, the amplitude of the EPSP progressively decreases, until it eventually inverts to a positive potential. If the stimulus current applied to the afferent fibers is increased, a negative potential develops superimposed on the positive EPSP, which reflects summated single-unit activity (Andersen et al. 1971) and is termed the population spike (PS). Such laminar analysis is used to study afferent distribution but lacks precision because of volume conduction of the large field potentials. We combined field potential and current source density (CSD) analysis in order to investigate the effect of CET on synaptic distribution in the hippocampus, since CSD analysis defines more precisely the distribution of synaptic currents. The theory of CSD analysis and the specific computational formulae have been presented in detail previously (Freeman and Nicholson 1975; Nicholson and Freeman 1975). The CSD is mathematically derived from extracellular field potentials by calculation of the second spatial derivative. Negative (inward) current flows reflect the location of a current sink, whereas positive (outward) current flows reflect the locus of a current source.

We first investigated the effects of CET on the distribution of Schaffer collateral-commissural (SCH/COM) afferents within the SR of rat hippocampal CA1 (Abraham et al. 1982). Stimulation of the SCH/COM afferents elicited short-latency, ne ative field potentials throughout the synaptic terminal zone (stratum radiatum). CSD analysis in normal animals revealed that the synaptic currents generated in the SR concentrate into bimodal yet overlapping components, peaking 71.3 μm and 228.3 μm from the pyramidal cell layer. CET produced: (1) a 13.2-percent shrinkage of the overall extent of current sinks in the SR; (2) a 37.4-percent reduction in the spatial extent of the sink proximal to the cell layer; and (3) an increase in the amplitude and area of the more distal sink. This contraction of proximal afferents and expansion of distal afferents was present even though the CSD profiles were adjusted to control for individual differences in the width of the major lamina of CA1. We tentatively proposed the proximal and distal sinks to reflect a separation of the COM and SCH afferents, respectively. CET thus appeared to have selectively produced persistent damage to the COM-CA1 pathway. This selective deafferentation combined with other, unspecified factors may have provided the stimulus for reorganization of SCH afferents (Abraham et al. 1982; Goldowitz et al. 1979).

We next investigated the persistent effect of CET on the distribution of entorhinal afferents to the stratum moleculare of the dentate gyrus (Abraham and Hunter 1982). Electrical stimulation of the angular bundle elicited a short-latency, negative field potential covering the outer two-thirds of the molecular layer. CSD analysis revealed that CET produced: (1) a significant shrinkage of the spatial extent of the current sink in the stratum moleculare; (2) a significant reduction in the distance from the peak

inward synaptic current to the granule cell layer; and (3) no change in the distance from the proximal inversion point to the granule cell layer. Taken together, these results indicate a loss of entorhinal afferents in the outer molecular layer. Coupled with available anatomical evidence, these results suggest that CET produces a preferential loss of the lateral entorhinal afferents to the dentate gyrus.

Anatomical Analysis of Afferent Synaptic Distribution in the Hippocampal Formation After CET

The foregoing results suggest that CET produces a specific reduction in COM afferents to CA1 and lateral entorhinal afferents to the DG. However, we do not yet know whether such results reflect a reduction in these specific afferent fiber populations, alterations in synaptic efficacy, or changes in the structure of target neurons. Therefore, we have begun the study of specific hippocampal afferents using morphological techniques. We have used acetylcholinesterase (AChE) (Geneser-Jensen and Blackstad 1971; Lynch et al. 1972) and Timm's sulfide-silver (Haug 1973; Zimmer and Haug 1978) histochemical stains and quantitative autoradiography (Fricke and Cowan 1978; Gottlieb and Cowan 1973) to assess alterations in the spatial extent and density of specific hippocampal afferents after CET. Quantitative analysis of this material is accomplished with a Computerized Image Analysis System linked to a microscope (King et al. 1985a; Reep et al. 1984).

The pattern of Timm's (Haug 1973; Zimmer and Haug 1978) and AChE (Geneser-Jensen and Blackstad 1971; Lynch et al. 1972) staining has been shown to correlate with the extent of a number of hippocampal afferents. In the molecular layer of the DG, for example, Timm's staining reveals three specific laminar bands that reflect the lateral entorhinal, medial entorhinal, and commissural/associational (C/A) afferents to the DG granule cells. The spatial extent (width) and the optical density of the Timm's stain bands were measured in the molecular layer of both blades of the right DG. CET did not significantly affect the widths of any of the bands in either blade. However, the stain density was significantly reduced by CET in each of the terminal fields of the buried blade, suggesting a reduced terminal density of lateral entorhinal and C/A afferents to the surviving granule cells. These results emphasize the importance of the quantitative analysis of stain intensity in these studies, since terminal density may be reduced by CET without a concomitant reduction in the spatial extent of the terminal field. Since the density of Timm's staining has not yet been quantitatively related to afferent terminal density, these results await confirmation by ongoing quantitative autoradiographic analysis of the effect of CET on the density of afferent terminals from the entorhinal cortex to the DG.

We have also completed analysis of the effect of CET on the pattern and density of AChE staining in the DG molecular layer contralateral to an entorhinal cortex lesion (the effect of CET on

the partially deafferented lesioned side will be discussed later). The DG molecular layer contralateral to an entorhinal cortex lesion is typically used as a within-subjects control against which alterations in the ipsilateral, partially deafferented DG molecular layer are assessed. As shown in table 1, CET produced significant reductions in the widths of the C/A clear zone (9 percent), the outer molecular layer (5 percent), and the total molecular layer (6 percent) in the contralateral buried blade. No significant effects of CET on AChE stain bands were observed in the exposed blade. In addition, CET produced significant increases in the density of AChE staining in both the C/A zone and the outer molecular layer of the buried blade and, to a lesser extent, of the exposed blade (table 2). Taken together, these results suggest that CET may have resulted in a reduction in the spatial extent and density of C/A and entorhinal afferents to the DG molecular layer, resulting in a compensatory expansion of cholinergic afferents. However, since these data were collected from animals receiving both prior CET and unilateral entorhinal cortex lesions, it is possible that the effects of CET on the contralateral molecular layer could be at least partially attributable to an interaction between CET and the entorhinal lesion. Consequently, we plan to examine the effect of CET on the pattern and density of AChE staining in rats not receiving entorhinal cortex lesions. We are also conducting autoradiographic studies to test more directly the hypothesis that CET produces a preferential loss of afferents from the lateral entorhinal cortex.

TABLE 1. *The effect of 10 weeks of ethanol treatment on the widths of AChE stain bands in the molecular layer of the DG contralateral to an entorhinal cortex lesion*

	Stain Band			
Group	SG	C/A	OML	ML
		Contralateral Buried Blade		
Ethanol (n=10)	44.7 (1.3)	*46.6 (1.1)	*158.6 (4.6)	*205.2 (3.1)
Sucrose (n=10)	43.9 (1.9)	51.1 (1.0)	167.3 (4.3)	218.4 (2.9)
		Contralateral Exposed Blade		
Ethanol (n=10)	42.1 (1.3)	46.1 (1.7)	183.1 (2.5)	229.2 (2.1)
Sucrose (n=10)	36.2 (1.5)	45.9 (1.7)	191.4 (5.6)	237.3 (3.5)

"p<.05

KEY: SG (supragranular band); C/A (commissural-associational); OML (outer molecular layer); ML (molecular layer).

NOTE: Values are the group mean (± S.E.M.) width in microns derived from individual means of 100 measurements per animal 110 measurements In each of 10 equally spaced horizontal sections through the hippocampus).

TABLE 2. *The effect of 20 weeks of ethanol treatment on the density of AChE staining in the DG contralateral to an entorhinal cortex lesion*

Group	Stain Band							
	SG	C/A	OML1	OML2	OML3	OML4	OML5	OML
				Contralateral Buried Blade				
Ethanol (n=10)	74 (3)	*125 (2)	*111 (2)	*102 (3)	*108 (4)	*130 (5)	*129 (5)	*116 (4)
Sucrose (n=10)	79 (3)	140 (3)	129 (5)	121 (5)	124 (6)	145 (6)	144 (7)	133 (6)
				Contralateral Exposed Blade				
Ethanol (n=10)	89 (4)	*140 (4)	*121 (5)	131 (5)	124 (6)	140 **(7)**	141 (6)	131 (5)
Sucrose (n=10)	99 (5)	151 (3)	134 (4)	141 (5)	135 (5)	143 **(5)**	140 (5)	139 (5)

*p<.05

KEY: SG (supragranular band); C/A (commissural-associational); OML (outer molecular layer).

NOTE: Values are the group mean (± S.E.M.) optical density (gray level) derived from Individual means of 100 measurements per blade for each animal 110 measurements in each of 10 equally spaced horizontal sections through the hippocampus). Lower values represent more dense staining, since gray levels of pixels can vary from 0 (black) to 255 (white). The outer molecular layer was divided into five equal portions for measurement (OML 1-5).

The Effect of CET on Synaptic Function in the Hippocampus: Electrophysiological Studies

Whereas the experiments described above provide us with important information in regard to the nature, extent, and specificity of the alteration and possible reorganization of hippocampal circuitry induced by CET, functional disturbances could be produced which are either undetectable by standard morphological methods or compensated for by adaptive mechanisms in a specific afferent system. In order to directly assess the functional alterations produced by CET, we are evaluating the synaptic strength (through input/output (I/O) functions) and synaptic plasticity (capacity for potentiation) in specific hippocampal afferents by means of extracellular field potential analysis.

We first studied the functional consequences of CET on the SCH/COM input to the SR of CA1 (Abraham et al. 1981). Eight weeks after CET, there was no significant alteration in the basic response strength to single shocks to the SCH/COM path (EPSP and PS measures) as assessed by varying the stimulus current (I/O curves); similarly, no changes were observed in the magnitude or duration of long-term potentiation (Alger and Teyler 1976) produced by 5-, 10-, or 100-Hz stimulus trains. However, CET significantly enhanced paired-pulse potentiation of PS amplitude without altering EPSP potentiation. Facilitation of the PS relative to control was also observed during brief trains of stimuli at 5 and 10 Hz (frequency potentiation). This latter pattern of results is similar to that found for a variety of treatments that reduce inhibition in the hippocampus (Lee et al. 1980). We therefore proposed that CET may produce a lasting disruption of intrinsic inhibitory neurotransmission in CA1 (Abraham et al. 1981).

We have recently evaluated the effects of CET on the response of DG granule cells to perforant path (from the entorhinal cortex) input (Abraham et al. 1984). CET did not influence the response strength or potentiation of basic synaptic responses (EPSP) but did decrease the PS. This effect could be seen in 4 ways: (1) in the asymptotic portions of the I/O curves, (2) in paired-pulse potentiation, (3) in the response to low-frequency stimulation (1 or 5 HZ), and (4) during the development of long-term potentiation. These results are consistent with previous anatomical evidence of granule cell loss after CET (Walker et al. 1980b), but they also suggest additional alterations such as a decrease in the effectiveness of the EPSP-spike coupling mechanism, an increased number or efficacy of synapses per surviving cell, or an increase in the spike threshold of DG granule cells surviving CET. Because these results in the DG are opposite (particularly for the paired-pulse series) to those we observed in CA1 (Abraham et al. 1981), it is obvious that CET does not uniformly influence all subfields of the hippocampus. This suggests that the degree of destructive and/or compensatory synaptic reorganization following damage by CET may differ depending on the specific subfield or afferent system studied.

When these studies were completed, we believed that CET reduced synaptic inhibition in CA1 by acting upon recurrent inhibitory processes (Abraham et al. 1981). Because considerable evidence has since accumulated to suggest the presence of feedforward as well as recurrent inhibition in CA1 (Buzaki 1984), we decided to reexamine these findings. We also decided to conduct these new studies using the in vitro hippocampal slice to avoid the use of anesthesia, because we thought it was possible that our previous in vivo results could have been complicated by an interaction of prior CET with urethane anesthesia (Abraham et al. 1981; Abraham et al. 1984). We used paired-pulse stimulation techniques, because they provide a sensitive way of separating feedforward and feedback inhibition. Antidromic conditioning stimuli provide a pure activation of recurrent inhibitory circuitry, whereas orthodromic conditioning stimuli should activate both feedforward and recurrent inhibitory processes (Rogers and Hunter 1985). Paired-pulse stimulation was delivered to CA1 in either orthodromic/orthodromic (O/O) or antidromic/orthodromic (A/O) pairs with interpulse intervals (IPI) ranging from 20 msec to 3 sec. CET reduces the inhibition of the PS test pulse response with A/O stimulation in a manner dependent upon current intensity. These results strongly support the conclusion that CET produces a persistent reduction of CA1 recurrent inhibitory processes--a conclusion also supported by intracellular recording in the hippocampal slice (Durand and Carlen 1984a). CET also enhanced the potentiation of the PS test response across a wide range of IPIs in response to O/O stimulation. That this was observed with conditioning pulses subthreshold for eliciting a PS (where activation of recurrent inhibition would be minimal) indicates that CET also reduces feedforward inhibitory processes. Taken together, this apparent reduction in both feedforward and recurrent inhibition indicates that CET produces an enduring disruption in the properties of inhibitory interneurons in the CA1 region.

Our most recent electrophysiological studies have sought to address the mechanism(s) underlying the action of CET on inhibition in CA1 (Hunter et al. 1986). We have examined, in the in vitro hippocampal slice, PS responses evoked by stimulation in the stratum radiatum (SR) or the stratum oriens (SO) in response to microiontophoresis of -aminobutyric acid (GABA) or bicuculline methiodide (BMI), a specific $GABA_a$ antagonist (Hunter et al. 1986). The sensitivity to GABA was evaluated by iontophoresis at 10 equidistant points parallel to the dendritic axis of pyramidal cells from the alveus to the hippocampal fissure. Because BMI had a prolonged duration of action, analysis was limited to four sampling points. We utilized drug concentrations, ejection duration, and backing currents derived from prior experiments which have utilized this procedure (Andersen et al. 1982; Andersen et al. 1980). When GABA was iontophoretically administered 6 msec prior to stimulation, CET produced an increase in the inhibitory response to GABA application in the stratum pyramidale (SP) and the SR with SR stimulation, as well as to application in the SP with SO stimulation. This increase in the inhibitory effect of GABA could also explain the apparent reduction in GABA facilitation

produced by application in the SO with SR stimulation and in the SR and SO with SO stimulation. BMI, a $GABA_a$ receptor antagonist, produced a profound increase in PS amplitude in response to SR and SO stimulation which peaked at approximately 1 minute following application and was greatest in magnitude with application sites near the SP. CET produced a significant increase in the excitatory response to BMI most pronounced with application in the SO and proximal SR. Though it is clear that this inhibition is mediated by GABA, the actions of GABA on CA1 pyramidal cells is quite complex, being mediated not only by $GABA_a$ and $GABA_b$ receptors but utilizing different ionic mechanisms in different parts of the cell as well (Alger and Nicoll 1982a; Alger and Nicoll 1982b; Andersen et al. 1980; Newberry and Nicoll 1984a; Newberry and Nicoll 1984b). Nevertheless, our results suggest that CET produces an enduring reduction in GABA release together with a compensatory up-regulation of $GABA_a$ receptor sensitivity or number. Our current working hypothesis is that this reduction of GABA release involves a CET-induced loss of GABAergic interneurons. However, enduring alterations in synaptic terminal excitability or GABA release cannot be ruled out, Further, potential CET-induced changes in depolarizing dendritic GABA receptors (Alger and Nicoll 1982b) and GABA receptors remain to be clarified. Indeed, Scheetz et al. (1986) reported that 3 months of CET reduced by 19 percent the number of GABAergic interneurons in the DG of a strain of ethanol-sensitive mice but did not change the number of these interneurons in a strain of ethanol-resistant mice. These findings also underscore the potential role of genetics in the susceptibility of the brain to the neurotoxic action of ethanol.

STUDIES OF POSSIBLE RECOVERY FROM ETHANOL-INDUCED DAMAGE IN SURVIVING HIPPOCAMPAL NEURONS

The extent and nature of possible reversibility of alcoholic brain damage is of considerable importance. Recent findings suggest that abstinent chronic alcoholics may recover significantly from both cerebral atrophy (Artmann et al. 1981; Carlen et al. 1984; Carlen et al. 1978; Ron et al. 1982) and cognitive deficits, though recovery of cognitive performance is significantly decreased by even moderate posttreatment alcohol consumption (Eckardt et al. 1980; Guthrie and Elliott 1980). Recent animal studies have supported the conclusion that recovery from CET-induced neuronal damage during ethanol abstinence can occur at both the morphological (King et al. 1985b; McMullen et al. 1984; Popova 1983) and functional (Durand and Carlen 1984b) levels.

We measured the density of dendritic spines on CA1 pyramidal and DG granule cells as one index of morphological recovery (King et al. 1985b). At the end of a 20-week CET period, half of the animals were sacrificed, and half were given a 20-week abstinence period before they were sacrificed. Brains were processed with a modified rapid Golgi method (Scheibel and Scheibel 1978) to stain dendritic spines, and spine density (spines/μm) was measured on the dendritic tree in the stratum oriens and stratum radiatum for CA1 pyramidal cells and in the stratum moleculare for DG granule

cells. CET without abstinence (group A0 vs. group S0) reduced the spine density of CA1 pyramidal cells and, surprisingly, increased the spine density of DG granule cells (table 3). After 20 weeks of abstinence (group A20 vs. group S20), the spine density had returned toward normal for both pyramidal cells and granule cells (table 3). There were no differences in spine density between the two control groups (S0 vs. S20) at any measurement location on either cell type. These results indicate an initial remodelling of spine density during CET that returns to normal during 20 weeks of ethanol abstinence. Kunz et al. (1976) previously reported that ethanol exposure for only 8 weeks produced an increased spine density in surviving CA1 pyramidal cells. This observation, coupled with our present results, suggests that the initial response of CA1 pyramidal cells to CET may be to increase spine density, which reverses to a decreased density during continued exposure and reverses again, toward normal, with abstinence. Whether granule cells respond to CET with an initial decrease followed by a compensatory increase in spines only after longer exposure remains to be determined.

TABLE 3. *The effect of 20 weeks of CET and 20 weeks of ethanol abstinence on the density of dendritic spines per linear micron of dendrites of CA1 pyramidal cells and DG granule cells*

Treatment Group	CA1 Pyramidal Cells		DG Granule Cells
	Stratum Oriens	Stratum Radiatum	Stratum Moleculare
Alcohol			
A0 (n=11)	1.779 (.079)	1.868 (.056)	**1.753 (.053)
A20 (n=8)	*2.010 (.045)	*2.181 (.046)	1.659 (.088)
Sucrose			
S0 (n=6)	1.892 (.079)	2.001 (.113)	1.571 (.058)
S20 (n=8)	1.873 (.115)	2.011 (.074)	1.538 (.145)

*p<.05 as compared to group A0.

**p<.05 as compared to group S0.

NOTE: Groups A0 end S0 (alcohol or sucrose) were killed after 20 weeks of treatment without an abstinence period. Groups A20 and S20 were killed 20 weeks after the end of the 20-week treatment period. Values are the mean (± S.E.M.) spines/μm.

Our recent Golgi results in the rat described above are at variance with our previous observations in the C57BL mouse of a

substantial spine loss from both CA1 pyramidal cell basal dendrites and DG granule cell dendrites after CET (Riley and Walker 1978). Our rat data are, however, in agreement with the recent report that dendritic spine density in CA1 is normal when assessed 3 or 6 months after CET (Lescaudron and Verna 1985). There are a number of possible explanations for the different results in mice and rats. The most likely include: (1) The mouse may simply be more sensitive to the toxic central nervous system (CNS) effects of CET than rats, particularly since the ethanol intake (g/kg) and resulting blood-ethanol levels are about doubled in the mouse relative to the rat. (2) The mouse hippocampus may possess less capacity for compensatory synaptic reorganization following neuronal injury. (3) Recent electron microscopic observations found that the dendritic spine heads of CA1 pyramidal cells in the mouse following CET were reduced in size relative to controls (Phillips and Cragg 1983). Many of the spine heads in the alcohol group were too small to be visible with the light microscope at standard magnification. Since our spine counts in the mouse study (Riley and Walker 1978) were made at a magnification of 1000X, it is possible that some of the spines in the alcohol group were not seen. Since our recent Golgi data in the rat were collected at a magnification of 2250X, this was not a problem, as demonstrated by the increase in spine density in granule cells after CET in the group without subsequent abstinence. The increase in spine density in granule cells is a possible anatomical substrate for the finding that CET reduced the PS amplitude in the DG elicited by stimulation of the entorhinal afferents, despite no change in the population EPSP (Abraham et al. 1984). That is, if the decreased number of surviving granule cells are innervated by an increased number of entorhinal synaptic terminals, fewer cells would be fired per unit EPSP, thereby reducing the PS amplitude although the EPSP amplitude remains unchanged.

THE EFFECT OF CET UN REACTIVE SYNAPTOGENESIS IN SURVIVING HIPPOCAMPAL NEURONS

The possibility that ethanol intake may limit morphological recovery from brain damage is suggested by reports that reversibility of cognitive performance and cerebral atrophy (Carlen et al. 1984; Eckardt et al. 1980) is significantly decreased by even moderate posttreatment alcohol consumption. We therefore conducted experiments to examine two hypotheses: (1) that prior CET results in a residual attenuation in the capacity for synaptic reorganization in response to brain injury; and (2) that CET during the period following brain injury suppresses synaptic reorganization. We tested these hypotheses by determining the effects of CET on the extent of reactive synaptogenesis of commissural/associational (C/A) and cholinergic afferents to DG granule cells following partial deafferentation induced by a unilateral lesion of the entorhinal cortex. We chose this model because it has been widely used to investigate reactive synaptogenesis (Cotman and Nadler 1978; Lynch and Cotman 1975). Unilateral ablation of the entorhinal cortex in adult rats removes 86 percent of the synapses from the distal 70 percent of the ipsilateral DG granule cell

dendrites (Matthews et al. 1976a). The synaptic reorganization and synaptogenesis subsequent to the denervation restores the partially deafferented terminal zone to its normal synaptic density (Matthews et al. 1976b). These new synapses originate primarily from the septal area, the commissural and associational hippocampal pyramidal cells, and the contralateral entorhinal cortex. The C/A fiber system expands outward by 10 to 20 μm, and the cholinergic septal fibers and fibers from the contralateral entorhinal cortex proliferate in the remainder of the denervated zone (Cotman and Nadler 1978). We have used histochemical staining for AChE to monitor these changes, because the expansion of the cholinergic septal input can be directly seen with this stain, and the expansion of the C/A system can also be measured because it appears as a "clear zone" devoid of any AChE stain (Nadler et al. 1980).

The first experiment of this series (King et al., in preparation; Walker et al. 1984) was designed to test the hypothesis that prior CET may result in a lasting attenuation in the capacity for reactive synaptogenesis in the DG. Two groups of rats were maintained on ethanol- or sucrose-containing liquid diets for 20 weeks. An 8-week abstinence period preceded partial denervation of the DG by unilateral electrolytic lesion of the entorhinal cortex. Horizontal sections were cut 40 days postlesion and processed for AChE staining. The width and optical density of afferent terminal fields were measured in the DG (King et al. 1985a). Unilateral lesion of the entorhinal cortex resulted in shrinkage of the ipsilateral outer molecular layer (OML) of both the buried (30 percent) and exposed (36 percent) blades of the DG. The shrinkage was almost identical in both CET and control groups, indicating that the lesion produced equivalent deafferentation. Despite equal lesion-induced removal of entorhinal terminals from the OML, the resulting expansion in the width of the C/A terminal field was reduced 50 percent in the buried blade and 33 percent in the exposed blade by prior CET. In addition, the condensation of AChE staining density in the OML ipsilateral to the lesion was reduced by prior CET, indicating that reactive synaptogenesis of cholinergic afferents to the DG granule cells was also residually suppressed by CET. These results indicate that prior CET produces a residual alteration in the capacity for synaptic reorganization that lasts at least 8 weeks after CET is discontinued. Using the same model, West et al. (1982) reported that 2 weeks of CET before, and 9 days of CET after a unilateral entorhinal cortex lesion almost totally blocked the expansion of the C/A fibers into the deafferented zone. These results suggest that postlesion ethanol exposure is even more effective than prelesion exposure in suppressing reactive synaptogenesis.

We have recently completed a study to further investigate the hypothesis that CET following brain damage suppresses synaptic reorganization (Orona et al., in preparation). In this experiment, the same model was used to study reactive synaptogenesis. except CET or control diets were administered for a 40-day period following a unilateral lesion of the entorhinal cortex in rats not

previously exposed to ethanol. The lesion-induced expansion of the ipsilateral C/A zone was reduced by greater than 50 percent as compared to controls. There was also less "clearing" of AChE staining density in the C/A zone and greater AChE condensation in the outer molecular layer. These results indicate that ethanol suppresses reactive synaptogenesis of C/A afferents following removal of the ipsilateral entorhinal afferents. CET may have also suppressed the expansion of the crossed entorhinal afferents arising from the contralateral entorhinal cortex, thus allowing the cholinergic afferents to expand more than normal.

Taken together, the results described above support the conclusion that both a prior history of ethanol exposure and ethanol exposure after brain damage limit the capacity of surviving neurons for recovery. These results have important implications for the treatment of brain-damaged alcoholics as well as nonalcoholic brain-damaged patients. Although the mechanism by which CET reduces reactive synaptogenesis is unknown, one possibility is an ethanol-induced elevation of glucocorticoids. Elevated glucocorticoid levels are known to inhibit both morphological and functional recovery following partial deafferentation (Dekosky et al. 1984), and ethanol exposure (both acute (Knych and Prohaska 1981) and chronic (Kakihana and Moore 1976; Tabakoff et al. 1978)) is known to increase the level of circulating glucocorticoids. Ethanol is also known to inhibit protein synthesis (Tewari et al. 1978). Since protein synthesis is undoubtedly required for reactive synaptogenesis, its inhibition by ethanol could limit the capacity for morphological reorganization.

CONCLUSIONS AND CAUTIONS

The foregoing results provide definitive evidence that prolonged ethanol exposure results in altered neuronal structure, connectivity, and function in the rodent hippocampus. Depending on the duration of ethanol exposure, the magnitude of exposure (blood-ethanol level maintained), and the genetic susceptibility of the species and strain, CET produces a loss of CA1 pyramidal and DG granule cells ranging from 10 to 30 percent (Irle and Markowitsch 1983; Kunz et al. 1976; Lescaudron and Verna 1985; Phillips and Cragg 1983; Roulet et al. 1985; Walker et al. 1980a; Walker et al. 1980b; Walker et al. 1981; Walker et al. 1982). The neurons surviving CET are also abnormal. Morphological studies indicate that surviving neurons possess fewer or rearranged synaptic contacts (Abraham and Hunter 1982; Abraham et al. 1982; Goldstein et al. 1983; King et al. 1985b; Kunz et al. 1976; McMullen et al. 1984; Riley and Walker 1978; Walker et al. 1981) (tables 1-3). Electrophysiological studies indicate that local inhibitory processes in area CA1 are deficient after CET (Abraham et al. 1981; Durand and Carlen 1984a; Rogers and Hunter 1985), perhaps due to loss of GABAergic interneurons (Scheetz et al. 1986). The capacity for long-term potentiation in CA1 (Durand and Carlen 1984b) and the DG (Abraham et al. 1984) is also reduced by CET. The available evidence also indicates that neurons surviving CET are capable of some degree of morphological (King et al. 1985b; McMullen et al.

1984; Popova 1983) and functional (Durand and Carlen 1984b) recovery during abstinence from ethanol , although this recovery is possibly limited by the suppression of reactive synaptogenesis by ethanol (King et al., in preparation; Orona et al., in preparation; Walker et al. 1984; West et al. 1982). Despite this recent accumulation of evidence demonstrating the neurotoxic effect of ethanol, many questions remain unanswered. The most important is the issue of why some cells are lost, and others are not. Neurons appear to be differentially susceptible to the toxic effects of CET both between and within cell types. For example, DG granule cells and CA1 pyramidal cells are apparently affected in an opposite manner both morphologically (King et al. 1985b) (table 3) and functionally (Abraham et al. 1981; Abraham et al. 1984). Further research into the mechanisms of this differential susceptibility of neurons to CET will therefore provide important clues to help determine how ethanol exerts its deleterious action on the brain.

Finally, we would like to discuss some of the issues that bear on the interpretation of data in these kinds of experiments. Because it is evident that both destructive and compensatory processes occur simultaneously during CET, caution must be used in attributing an observed morphological or functional alteration as representing solely a destructive or a compensatory mechanism. or in interpreting a negative result as evidence of the absence of an effect. During CET, for example, hippocampal cells are killed or damaged, resulting in a partial deafferentation of other neurons, which leads in turn to reactive synaptogenesis in undamaged afferents. The end result may be that the total number of synapses innervating a specific neuronal population may not differ from controls, but the afferent synapses on the surviving cells will have been rearranged and therefore differ qualitatively. Simply counting the number of synapses in a particular region without determining their origin may thus lead to the erroneous conclusion that CET did not have an effect.

Another important issue is the interpretation of quantitative cell count or synaptic density data after CET. A loss of cells or synapses can be masked by a change in total area or volume of a cell population or a synaptic field. In a previous study, for example, we found that DG granule cell packing density (nucleoli per unit area in a grid) was not significantly altered by CET, but the total area of the granule cell layer was reduced by about 20 percent as a result of a significant overall decrease in cell number (Walker et al. 1980b). The same phenomenon has been observed in the cerebellum (Tavares and Paula-Barbosa 1982; Walker et al. 1981). Finally, the importance of conducting parallel anatomical and functional analyses of the effects of CET should be emphasized. In the spinal cord, for example, axonal sprouting can lead to the development of abnormal function (McCouch et al. 1958). Compensatory synaptic reorganization during or following CET thus may or may not promote functional recovery. Even in the absence of morphological recovery or compensation, surviving afferent connections could exhibit changes in synaptic efficacy or potency which would not be detected morphologically.

It is apparent that, despite the accumulation of substantial information during the last few years regarding the effects of prolonged ethanol exposure on neurons, a great many questions remain to be answered. Clearly, the results obtained to date reveal that the toxic effects of ethanol are dynamic and much more complicated than suspected. Similarly complex dynamics may be expected to result from the toxic effects of other drugs.

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Amphetamine Changes Neostriatal Morphology

Philip M. Groves, Lawrence J. Ryan, and Jean C. Linder

INTRODUCTION

The mammalian neostriatum, consisting of the caudate nucleus and putamen, is known to be a principal site of dysfunction in Huntington's and Parkinson's diseases. On the basis of this and substantial other evidence, the neostriatum is believed to play an important role in both motor and cognitive functions of the human brain (Groves 1983). It has also been repeatedly established that the neostriatum is an important site of action of many psychoactive drugs, including amphetamine, cocaine, and opiates.

It is now clear that amphetamine acts on one of the major inputs to the neostriatum (the dopaminergic system arising from the substantia nigra, pars compacta) to release dopamine onto postsynaptic targets. Amphetamine also alters the firing rate of the presynaptic dopamine neurons and releases dopamine from their dendrites, leading to the phenomenon of self-inhibition (Groves et al. 1975). Dopamine released within the neostriatum also has a prominent site of action at autoreceptors located on dopaminergic neuron terminals. Here it can regulate the amount of dopamine released by each nerve impulse and alter biophysical properties of the synaptic endings, for example, the electrical excitability of the terminals (Tepper et al. 1985).

Over 95 percent of neurons within the neostriatum belong to a single morphological class: the neostriatal medium spiny neuron. This cell type is distinguished by its highly stereotyped morphology and medium size. Three to five primary dendrites usually radiate from the soma in a stellate pattern to form a spherical dendritic field approximately 500 microns across. The dendritic branching is distorted at the boundaries of histochemically defined subregions (the striosomes and matrix (Graybiel and Ragsdale 1983)), so that neostriatal cells are confined to one anatomical compartment (Penny et al. 1984). The cell body and proximal dendritic trunks are smooth, but at 20 microns from the soma, the dendrites abruptly become heavily invested with dendritic spines, ranging in density from one to four spines per micron of dendritic

length (Wilson and Groves 1980). Each spine receives one or more synaptic inputs arising from the cerebral cortex, midline thalamus, and substantia nigra. In addition, neostriatal inputs arise from several other telencephalic and brain stem afferent systems, including the basolateral amygdala and the raphe nuclei. Each spine consists of a typically spherical head approximately 0.1-0.9 microns in diameter connected to the parent dendrite by a shaft approximately 0.4-3.8 microns in length (Wilson et al. 1983). Synapses from the major excitatory inputs of the thalamus and cerebral cortex form asymmetrical (Gray's type I) synapses onto the heads of the dendritic spines. Less commonly, a symmetrical (Gray's type II) synapse is made onto the spine shaft. Despite the use of a wide variety of anatomical approaches, including anterograde degeneration, autoradiography (Hattori et al. 1973), 5-hydroxydopamine labelling (Groves 1980; Arluison et al. 1978a), radiolabelled dopamine uptake (Arluison et al. 1978b), and immunocytochemical identification of tyrosine hydroxylase immunoreactivity (Freund et al. 1984; Pickel et al. 1981), a definitive identification of the synapses made in the neostriatum by dopaminergic projections arising from the brain stem has yet to be made.

ALTERATIONS INDUCED BY REPEATED AMPHETAMINE ADMINISTRATION

Repeated administration of amphetamine leads to a number of long-lasting behavioral, physiological, and biochemical changes, indicating the inherent plasticity of the neostriatum and its dopamine input. For example, the locomotor and stereotyped behaviors induced by acute amphetamine administration show a progressive augmentation with repeated administration of the drug (Segal and Schuckit 1983). This sensitization may parallel the progressive augmentation of the psychotomimetic properties of this drug in human amphetamine abusers. Similar behavioral effects have been demonstrated with repeated administration of cocaine.

Repeated administration of amphetamine has also been demonstrated to produce substantial biochemical and morphological alterations in the neostriatum. For example, following the repeated administration of amphetamine over several days, there is a substantial and persistent decline in neostriatal dopamine content in a variety of experimental animals (Ellison et al. 1978; Seiden et al. 1975/76; Wagner et al. 1980a). Indeed, even following a single high dose of the drug in rats pretreated with iprindole to retard the degradation of amphetamine, neostriatal dopamine content may be reduced for up to 1 year (Fuller and Hemrick-Luecke 1980). Such long-lasting changes have also been demonstrated with chronically administered dopamine antagonists; in humans, dopamine-antagonist-induced motor disturbances may persist for 5 years or longer (Marsden 1982). Other examples of biochemical plasticity following chronic amphetamine administration include a decreased number of dopamine uptake sites (Wagner et al. 1980b) and a decline in neostriatal tyrosine hydroxylase activity (Hotchkiss and Gibb 1980).

Dramatic morphological changes accompany these biochemical sequelae of chronic amphetamine administration. Individual dopaminergic axons in the neostriatum, revealed by histofluorescence, appear to be swollen and distorted, and background fluorescence appears to be reduced, suggesting a reduction in dopamine content (Ellison et al. 1978; Jonsson and Nwanze 1982; Lorez 1981; Nwanze and Jonsson 1981). This reduction probably reflects degeneration of dopamine axonal terminals, since silver stains (such as the Fink-Heimer method), used to reveal degenerating axons and their terminals, show significant axonal degeneration in the neostriatum following chronic amphetamine administration (Ricaurte et al. 1982; Ricaurte et al. 1984a; Ricaurte et al. 1984b; Wahnschaffee and Esslen 1985). This damage appears to be rather specific, because degeneration was not observed in several other brain regions, including the dopaminergic substantia nigra and ventral tegmental area, the noradrenergic locus coeruleus, and the serotonergic raphe nuclei. In immature guinea pigs, chronic administration of amphetamine has also been reported to lead to axonal degeneration in circumscribed regions of the frontal neocortex (Wahnschaffee and Esslen 1985). Striatal dopaminergic axons seem particularly vulnerable to drug-induced damage, because axonal degeneration also occurs with administration of the neurotoxic contaminant (1-methyl-4-phenyl-1,2.3,6-tetrahydropyridine, commonly known as MPTP) found in certain synthetic street preparations of abused drugs. Ingestion of this compound leads to dopamine cell loss in the substantia nigra and to development of a Parkinsonian syndrome in humans and several other species (Markey et al. 1986).

Thus, it has become clear that amphetamine can be neurotoxic when administered repeatedly at high doses. Gross degenerative changes are, in one sense, easily observable plastic changes that may be taken to suggest that more subtle morphological alterations can occur at lower doses and with less frequent administration. Furthermore, since these neurodegenerative changes have been demonstrated in albino rats, other animals, more sensitive to the neurotoxic actions of amphetamine, may show different patterns and types of changes. The importance of multispecies comparisons and use of pigmented experimental animals for studying neurodegenerative changes has recently been highlighted by the fact that the neurotoxic actions of MPTP could not be demonstrated in albino rats (Markey et al. 1986).

STUDIES OF THE EFFECTS OF CHRONIC AMPHETAMINE ADMINISTRATION

Approaches/Procedures

Our studies of amphetamine-induced plastic changes have entailed analysis of both gross degenerative changes and more subtle anatomical alterations. Some of our results illustrate the potential of the neostriatum to alter its morphology in response to repeated drug insult. Such morphological changes are presumed to alter neostriatal cognitive and motor functions. Our studies have used several anatomical approaches, including tyrosine hydroxylase

and leu-enkephalin immunocytochemistry, Fink-Heimer degeneration, and electron microscopy, to analyze the neurotoxic effects of chronically administered amphetamine on the brains of pigmented Long-Evans black-hooded rats. We view these studies as complementary to our continuing studies of more subtle morphological and functional alterations induced by chronically administered amphetamine on the structure and function of presynaptic endings and postsynaptic targets of the dopaminergic projections into the neostriatum.

d-Amphetamine sulfate was continuously administered to rats for periods of 1 to 3 days via indwelling subcutaneous minipumps (Alzet minipumps, model 2ML1). For some electron microscopic studies and Fink-Heimer staining, animals were sacrificed and perfused immediately at the end of drug administration. For the immtunocytochemical and other Fink-Heimer staining experiments, animals survived for 1 day prior to perfusion. Standard anatomical procedures were followed throughout.

Results

Our experiments confirm the general outline of neurotoxic amphetamine effects identified by Schuster, Seiden, and their colleagues (Ricaurte et al. 1982; Ricaurte et al. 1984a; Ricaurte et al. 1984b) and demonstrate several other important effects. For example, in pigmented animals, the degenerative sequelae are more extensive, involving large regions of motor and somatosensorytortex as well as the neostriatum. Within the neostriatum, extensive degeneration, involving most of this structure, is seen with Fink-Heimer labelling (the most posterior regions usually show the least degeneration). In figure 1, the deposition of silver grains, indicative of axonal degeneration, is clearly seen in the neostriatum of a Long-Evans rat treated with approximately 60 mg/kg/day of d-amphetamine sulfate for 3 days. The effect is dose related; doses ranging from 15.9 to 67.6 mg/kg/day show progressively greater and more extensive degeneration. Behavioral effects were also dose related; the lowest doses of the drug produced increased locomotion, hyperresponsiveness, and moderate weight loss, and the highest doses led to intense and continuous stereotyped grooming, orofacial stereotypy, severe weight loss, and insomnia.

These dramatic degenerative and behavioral consequences of chronic amphetamine administration are thought to result from the release and accumulation of dopamine. The neostriatal degeneration observed as a result of the chronic amphetamine regimen probably reflects damage to dopaminergic afferent axons, because previous studies using histofluorescence have revealed numerous swollen fluorescent axons (Ellison et al. 1978; Lorez 1981; Nwanze and Jonsson 1981), and, in our tissue, we have found many abnormally swollen and distended axons labelled immunocytochemically for tyrosine hydroxylase. Swollen profiles such as those shown in figure 2 were not found in sham-operated control rats.

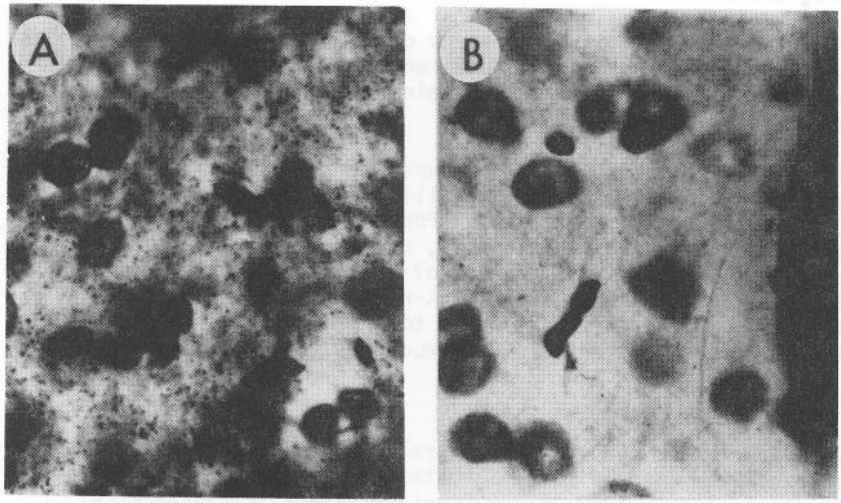

FIGURE 1. *Neostriatal axonal terminal degeneration*

NOTE: (A) Axonal degeneration, as shown by dense silver grain deposits. is seen throughout this section of neostriatum from a Long-Evans rat that received 60 mg/kg/day d-amphetamine sulfate for 3 days. (B) No degeneration is seen In a sham-operated control rat.

The distortion of dopamine axons was specific to the neostriatum. In the globus pallidus, which also possesses a dense network of tyrosine-hydroxylase-immunoreactive axons, neither swollen and distorted immunolabelled axons nor Fink-Heimer-labelled degeneration was found. In the electron microscope, neostriatal tissue had numerous profiles showing classical signs of degeneration. The profile shown in figure 3, for instance, is electron dense (dark) and shows scalloped and shrunken margins. Many vesicle-containing profiles appeared distended and filled with debris. These perhaps correspond to the swollen axons seen in the light microscope.

Labelling, either for Fink-Heimer axonal degeneration or for tyrosine hydroxylase immunoreactivity, failed to reveal axonal or somatic degeneration or damage in other regions of the basal ganglia, including the substantia nigra, globus pallidus. and subthalamus, nor was damage observed in any cortical region receiving a dopaminergic projection. Damage was not observed in the noradrenergic nucleus locus coeruleus nor in some targets of the locus coeruleus, including the hippocampus, cerebellum, and thalamus. In the thalamus, the midline nuclei, which project into the

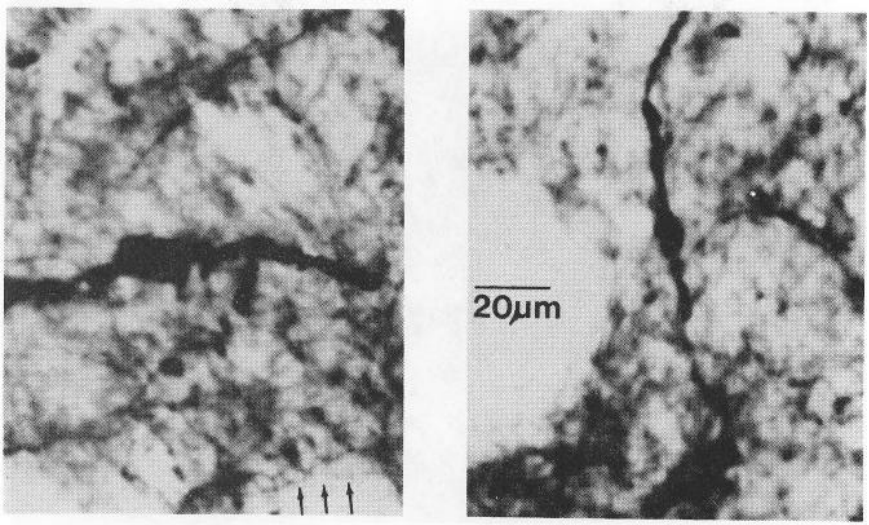

FIGURE 2. *Neostriatal axonal distortions*

NOTE: Large swollen and distorted axons are seen labelled for tyrosine hydroxylase immunoreactivity in the neostriatum of a rat treated for 3 days with 50 mg/kg/day d-amphetamine. Normal axons at arrows.

neostriatum, and the ventral nuclei, which receive output from the basal ganglia and motor cortex, were examined with particular care. No damage was observed in these regions following any regimen of chronic amphetamine administration.

In pigmented Long-Evans rats, extensive degeneration was observed in frontal cortex. This damage was densest in regions of primary motor cortex and supplementary motor cortex, with lighter terminal degeneration in sensorimotor cortex, though only circumscribed parts of these regions were involved. Furthermore, animals that showed greater behavioral effects and greatest weight loss showed the most extensive neocortical damage. Figure 4 illustrates the prominent yet circumscribed nature of the damage to motor cortex. The densest degeneration within motor cortex appears to be in intermediate layers, though Fink-Heimer labelling is seen throughout the depth of the cortex. Whether or not the degeneration represents monoaminergic elements is a topic of current investigation. It seems unlikely, though, that dopaminergic terminals are involved, because these regions are not known to receive a dopaminergic innervation.

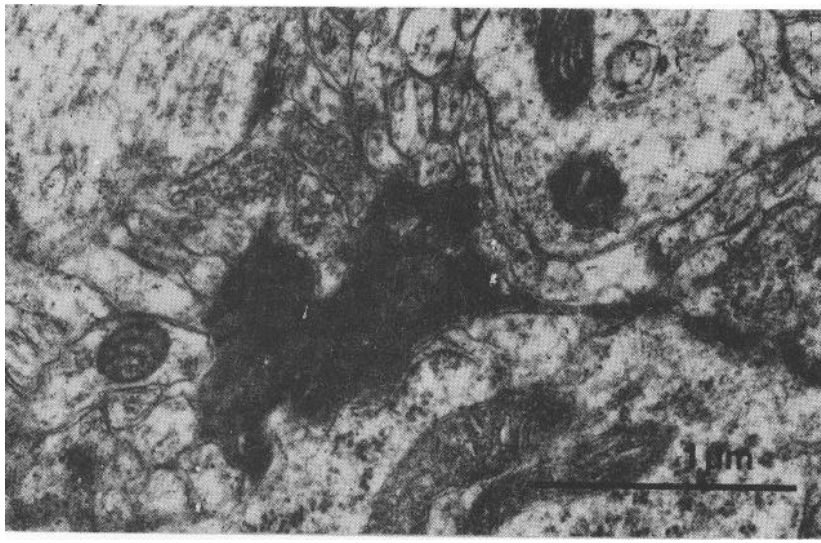

FIGURE 3. *Ultrastructure of neostriatal degeneration*

NOTE: The figure shows an electron micrograph of a typical dark, degenerating profile In the neostriatum of a rat treated with 50 mg/kg/day of d-amphetamine. The profile is debris filled, with scalloped. distorted edges.

Neocortical degeneration was not observed in two albino Sprague-Dawley rats in which degeneration was observed in the neostriatum, nor has extensive neocortical damage been reported in earlier studies of repeated amphetamine administration in other albino rats (Ricaurte et al. 1982; Ricaurte et al. 1984a; Ricaurte et al. 1984b). The only previous report of dense cortical degeneration was in pigmented guinea pigs, but, since immature animals were used in those experiments Wahnschaffee and Esslen 1985). the authors suggested that cortical damage resulted from a greater vulnerability of the immature nervous system to amphetamine than is seen in adults. We suggest that the presence of pigmentation may also be an important variable.

More subtle alterations in neostriatal morphology may also be produced by chronic amphetamine administration. The neostriatum possesses several distinct anatomical compartments defined by histochemical and connectional criteria (Graybiel and Ragsdale 1983; Gerfen 1985). One compartment, termed the striosomes, stains heavily for substance-P, leu-enkephalin, opiate receptors, and, in immature rat brains, for tyrosine hydroxylase. Medium spiny neurons within the striosomal compartment appear to project

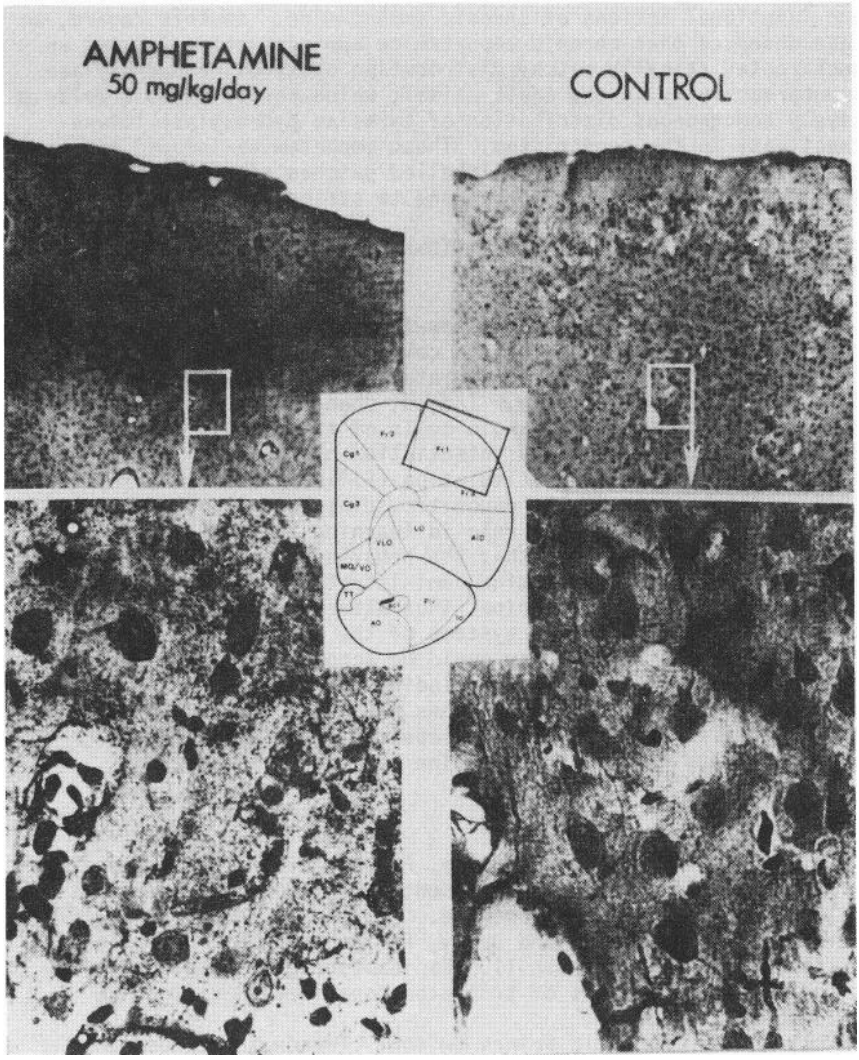

FIGURE 4. *Dense degeneration in a circumscribed region of primary motor cortex following 3 days of continous amphetamine administration*

into the dopaminergic substantia nigra, pars compacta (Gerfen 1985). The larger matrix compartment, in contrast, characteristically labels more heavily for acetylcholinesterase and somatostatin, and projects into the substantia nigra, pars reticulata (Gerfen 1985). The existence of two distinct compartments opens

the possibility of an anatomical basis for differential plastic and functional actions of chronic amphetamine. In this regard, we have observed that chronic amphetamine administration induces an uncharacteristically patchy distribution of tyrosine hydroxylase immunoreactivity in the adult animal, which normally has a relatively homogeneous distribution of tyrosine hydroxylase immunoreactivity in the neostriatum. These amphetamine-induced patches correspond to leu-enkephalin-labelled patches seen in adjacent sections and so probably correspond to striosomes.

POSSIBLE DENDRITIC SPINE ALTERATIONS RESULTING FROM CHRONIC AMPHETAMINE ADMINISTRATION

In addition to these large, systemwide changes, it is conceivable that the dendritic spines of the common spiny neuron, where the three major inputs to the neostriatum synapse, represent a site for drug-induced plasticity. Indeed, changes in the morphology of the dendritic spines of granule cells of the dentate gyrus have been suggested to accompany potentiation of synaptic transmission following high-frequency stimulation (Fifkova and Van Harreveld 1977). Alterations in the dendritic spine have also been theorized to play an important role in learning and memory (Crick 1982). It is conceivable that the behavioral augmentation seen following repeated amphetamine administration results from similar changes at the dendritic spine, thus altering the synaptic efficacy of the major afferent systems of the neostriatum. Such changes in synaptic efficacy could be accompanied by changes in the morphology of the spine, including the size and shape of the synaptic contact. Such alterations are currently being examined and quantified in animals given repeated administrations of amphetamine as well as the dopamine antagonist haloperidol.

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Delta-9-Tetrahydrocannabinol-Dependent Alterations in Brain Structure

Philip W. Landfield

INTRODUCTION

Although chronic exposure to marijuana or cannabinoids has been found to induce lasting behavioral changes (Nahas and Paton 1979; Stiglick and Kalant 1982), little is known about the nature of the neural consequences of such long-term exposure. In particular, it is not known whether long-term use induces structural changes in the brain (which presumably are only gradually reversible, if at all) and, if so, whether such anatomical changes are associated with functional alterations. It is clear that cannabinoids strongly affect central neural function (cf. Turkanis and Karler 1983; Campbell et al. 1986). but there have been essentially no major systematic studies of the long-term effects on brain structure of chronic marijuana exposure, or of exposure to delta-g-tetrahydrocannabinol (THC), the primary active constituent of marijuana. One research project on this question (Harper et al. 1977; Heath et al. 1979) reported ultrastructural brain changes in monkeys exposed to marijuana and THC, but the groups included only 2 to 3 animals. Furthermore, some experimental (but not control) animals bore implanted depth electrodes, and several of the ultrastructural changes reportedly induced by THC, e.g., clumped vesicles and large intercellular spaces, are characteristic of poor tissue fixation.

Thus, apart from the studies conducted at this laboratory, which are described below (see Landfield et al. (1987) for a more detailed description), and one other ongoing project with rats (e.g., Scallet et al. 1986). almost nothing is known about the effects of chronic THC exposure on mammalian brain structure.

Clearly, there are important sociomedical reasons for conducting studies to determine whether chronic THC exposure induces changes in brain anatomy. Moreover, assessing the effects of THC might be valuable in the analysis of basic neurobiological processes, since a substance able to alter brain structure in adult mammals would be of considerable interest in this regard. Consequently, we have

undertaken extensive, long-term studies in rats, using quantitative light and electron microscopic approaches, to assess the effects of chronic THC exposure on brain anatomy. The present chapter summarizes the rationale and results to date of these studies, and also considers possible interactions of THC with endogenous adrenal steroid systems. As discussed below, the increasing evidence that such interactions occur, in conjunction with evidence that corticosteroids are able to modulate brain structure in adult mammals, provides some of the stronger indications of a possible effect of chronic THC on brain structure.

INTERACTIONS OF THC AND GLUCOCORTICOID SYSTEMS

Corticosteroid Effects on Hippocampal Correlates of Aging

The mechanisms of aging in general, and brain aging in particular, are not well understood, but there is growing evidence that neuroendocrine factors play at least a partial role in the vertebrate aging processes (cf. Adelman and Roth 1982; Finch 1976; Finch and Landfield 1985; Simpkins and Estes 1983). We have previously proposed that glucocorticoids accelerate the process of brain aging (Landfield 1978; Landfield et al. 1978; Landfield et al. 1981a), because some pathological effects of corticoids in nonneural systems are overtly similar to changes seen with normal aging (cf. review in Wexler 1976), and because the hippocampus, which is one of the brain structures most affected by aging (Brody 1973; Wisniewski and Terry 1973), contains the highest density of specific corticosteroid receptors of any brain structure (McEwen et al. 1975). The hypothesis that hormones modulate brain aging led to several directly testable predictions, including: (1) that the activity of the adrenal system should correlate quantitatively with measures of brain aging, and (2) that experimental manipulation of adrenal steroids should modify the rate of appearance of hippocampal correlates of aging. Further, if adrenal steroids normally influence a component of brain aging (cf. Landfield 1978). then prolonged adrenalectomy should be able to retard this process.

Testing each of these predictions, however, required the use of reliable and sensitive indices of brain aging, which were not then readily available. We therefore conducted an extensive series of morphometric analyses of age-related changes in a well-defined region of the rat hippocampus (field CA1), at both light and electron microscopic levels (Applegate et al. 1984; Landfield et al. 1981b; Lindsey et al. 1979). Using this model system and these measures, we tested the above predictions and found substantial evidence consistent with the hypothesis (e.g., Landfield et al. 1978). In particular, we found that adrenalectomy could retard the development of several light microscopic morphologic indices of hippocampal aging (Landfield et al. 1979; Landfield et al. 1981a), supporting the view that normal levels of corticosteroids participate in brain aging. Other studies have found that elevated corticosterone accelerates the decrease in neuronal density seen with aging (Sapolsky et al. 1985).

Although the evidence that corticosteroids influence brain aging appears reasonably sound and consistent, there is still very little known about the mechanisms of these effects. Changing corticosteroid levels alters many other hormonal systems, including ACTH and related neuropeptides. These peptides can exert a direct (stimulantlike) action on some measures of brain physiology, chemistry, and behavior (Gispen et al. 1977; Pfaff et al. 1971; Renaud and Padjen 1978). To test the possibility that prolonged adrenalectomy might have retarded brain aging correlates by elevating ACTH and, consequently, increasing neural activation, we conducted long-term studies of the effects of daily administration of an ACTH analog, ORG 2766, and of an established neural stimulant, pentylenetetrazol. These agents retarded some brain aging correlates, as did prolonged adrenalectomy (Landfield et al. 1981a). Therefore, although the pattern of effects differed somewhat from that of adrenalectomy, the data raise the possibility that more than one hormonal system may influence brain aging. Nevertheless, it has been found recently that specific corticosteroid-receptor-bearing neurons appear to be preferentially lost from the hippocampus during aging or following chronic administration of corticosterone (Sapolsky et al. 1985), which lends considerable support to the view that corticosteroids act on brain aging by a specific corticoid-receptor-mediated effect.

Effects of THC on Corticosteroids

It has been known for some years that THC or marijuana extract can induce alterations in a variety of endocrine systems, including an increased activity of the adrenal-pituitary axis (Dewey et al. 1970; Drew and Slagel 1973; Maier and Maitre 1975; Rosenkrantz et al. 1975; see reviews in Braude and Ludford 1984). The latter increase has been seen frequently in rats, and we also found an increase of ACTH and corticosterone under our chronic treatment conditions (see below). This increase, however, has not been observed consistently in the few human or monkey studies that have examined this question (e.g., Kolodny et al. 1974). On the other hand, the adrenal-pituitary axis is extremely labile and difficult to assess, and thymus involution, which could imply increased corticoid activity, has been reported in monkeys given 15 days of THC treatment (Thompson et al. 1974). More complete studies are needed, e.g., on the 24-hour diurnal rhythm of cortisol, to obtain a clear-cut answer to the question of human adrenal-pituitary axis responses to THC.

Potential Corticoidlike Mechanism of the THC Effect on Brain

Evidence that THC increases adrenal-pituitary axis activity, in conjunction with evidence that glucocorticoids accelerate some aspects of brain aging, clearly raises the possibility that any effects of THC on brain structure might be mediated in part by an elevation of steroid activity. However, it is not necessary to postulate that the THC influence on brain is mediated by endogenous corticoids in order to consider the possibility that THC acts on brain via steroidlike mechanisms. Because THC and some of its

metabolites have chemical structures similar to those of some naturally occurring adrenal steroids (e.g., Harris et al. 1976; Szara 1973), it seems conceivable that THC could act on the brain directly, perhaps by mimicking glucocorticoids and binding to glucocorticoid receptors in the hippocampus. Other investigators have raised the possibility that THC may bind to estrogen receptors (Rawitch et al. 1977; Solomon et al. 1976) or induce estrogenlike effects in the brain (Foy et al. 1982). However, not all workers have found data consistent with this effect (Okey and Bondy 1978).

Clearly, it is not yet established that THC acts on the brain through glucocorticoidlike mechanisms. However, if this route of action eventually proves to be the basis of the THC effect, then, regardless of whether THC acts on hippocampal morphology directly (by mimicking glucocorticoids) or indirectly (by elevating endogenous adrenal steroids), the outcome of THC actions might be to accelerate the development of a steroid-dependent aginglike pathology in the brain. Young animals may be able to adapt somewhat to putative THC-induced aginglike effects, but, as adaptive capacity declines with age, the degree of brain pathology seen during aging might be substantially greater in animals previously exposed to THC than in nonexposed animals. Thus, chronic use of marijuana in youth could have implications for the quality of later life that have not yet been recognized.

Based on these considerations, the studies described below were aimed at defining the nature and possible underlying mechanisms of the effects on brain structure of chronic THC exposure. The studies were conducted in the rat hippocampus, in which morphology has been found to be affected by corticoid exposure, with treatment periods sufficient to induce gradual, possibly aginglike effects on the brain (e.g., approximately 30 percent of the lifespan: 8 months). Systematic, quantitative analyses of brain morphology were performed, and direct measures of the chronic treatment effects on adrenal-pituitary system hormones were carried out.

LONG-TERM STUDIES

Subjects and THC Administration

Male, Fischer 344 rats, 3 to 4 months old at the start of the study, were housed doubly in an animal facility containing an HEPA (high-efficiency particulate; absolute) filter/laminar air flow system which filtered all particles larger than 0.3 from from the air. The air filter removed airborne bacteria, and no infections developed in the colony. Administration of agents was carried out by an investigator wearing a clean lab coat and surgical mask and gloves, using sterile needles.

The basic protocols used for preparation of THC are similar to those described by Smiley et al. (1976). Briefly, the THC was suspended in a solution of detergent (Pluronic F68; Wyandotte Chemical Company) and ethanol. The ethanol was subsequently

evaporated, saline was added to make up the appropriate concentrations, and the solutions were sonicated.

Pathology and Blood Chemistry Studies

Animals were injected subcutaneously (SC) 5 times weekly with saline alone, the Pluronic F68 vehicle alone, or 8 mg/kg THC in Pluronic, for a period of 4 months. Blood samples were obtained from each animal after 2 months and after 4 months of treatment (by tail sampling while the animals were held in a restraining cage). On each sampling occasion, blood was collected at both 2 minutes and 12 minutes after the animal was first handled and placed in the restraining apparatus.

The animals were sacrificed after the 4-month test, and a complete blood chemistry test (SMAC) was performed on plasma. The saline and Pluronic-vehicle-alone groups did not differ from each other on any variable. However, the THC group exhibited significant elevations in glucose, cholesterol, and perhaps creatinine and uric acid. Other investigators also have found THC-induced alterations in glucose and cholesterol levels (e.g., Rosenkrantz et al. 1975). In addition, plasma from THC animals showed significant decreases in total protein and albumin. Interestingly, several of these changes are similar to those found in normally aging rats (Landfield, unpublished results).

In the endocrine assays, THC-treated rats exhibited significant elevations in plasma ACTH and corticosterone. In particular, ACTH was dramatically increased after 12 minutes of restraint stress. Thus, these results confirm earlier reports of chronic THC-induced increases in adrenal activity and also provide direct evidence that the effect is due to elevated ACTH. The negative feedback control of ACTH by corticosterone has been postulated to be disrupted by THC (Drew and Slagel 1973), an effect which would be consistent with the steroidlike structure of THC (Szara 1973). Similar elevations of stress-induced levels of ACTH and corticosterone are seen during aging (e.g., Landfield et al. 1978; Lewis and Wexler 1974; Sapolsky et al. 1983).

Long-Term Morphometric and Behavioral Study

Similar long-term studies were conducted in other groups of young rats, to investigate the possible morphological and behavioral effects of chronic THC on the brain. These groups included a Pluronic-vehicle-alone group (n=12), a low-dose THC (4 mg/kg) group (n=12), and a high-dose (10 mg/kg) THC group (n=12). The high dose was reduced to 8 mg/kg approximately 1 month after the start of these studies, because the animals appeared to react as if the treatment were aversive.

Both the low and high doses of THC caused slight reductions in body weight during chronic treatment. This reduction apparently was due to decreased food and water intake, although the latter effects were mild. We tested these variables on four occasions

during the 8-month protocol. Water intake was found to be significantly reduced in THC animals, but the reduction of food intake was not quite statistically significant. Of interest from the point of view of a possible endocrinelike effect of THC, is the fact that chronic treatment of rats with glucocorticoids induces very similar, but more severe effects on body weight and food intake (Stevenson and Franklin 1970).

Behavioral studies. On two occasions during the 8-month treatment regimen, animals were examined for open field behavior, and at two other times, they were tested for learning in a simple, one-trial, active-avoidance paradigm. On the first test, animals were given four retention/training trials at one trial per day (spaced trials), and, on the second test, they were given three trials on one day, each separated by 1 minute (massed trials).

Fourteen days following cessation of THC treatments, all animals were assessed for acquisition and retention of maze-reversal learning, which is affected both by hippocampal damage and aging. Animals were trained for 1 week to consistently choose one arm of a T-maze (to avoid footshock). The safe side was then reversed, and the animals were trained to select the opposite side on three successive acquisition trials (acquisition data were analyzed only for the second and third trials, since the first trial was always incorrect). They were then tested for retention of the reversal 24 hours later, on three retention trials. Animals were then reversed again using the same trial sequence.

The high dose of THC exerted moderate effects on open field activity and active-avoidance training, but only on the first trial of each paradigm. That is, after the first retention trial of the spaced active-avoidance trials, THC animals matched the performance of the controls. In addition, THC animals were no different from controls on any of the trials of the subsequent massed active-avoidance test at the end of 8 months. Moreover, THC animals only differed from controls on the first test of open field activity (although habituation affected scores of the controls after the first session). Similarly, no differences in choice or latency were found among groups in performance of the T-maze reversal 2 weeks after the end of the THC treatments.

These very moderate effects of chronic THC on behavioral performance suggest that 8 mg/kg SC was not an effective high dose for rats, at least under chronic conditions in which metabolism, e.g., clearance, and behavioral processes may be able to adapt. However, it is also highly possible that the tasks used to test the animals were too simple or were not appropriate for detecting effects of THC.

Neuroanatomical methods. Following the behavioral tests, animals were deeply anesthetized and perfused intracardially with an aldehyde solution. The brains were then prepared and trimmed for light and electron microscopy by methods outlined below.

All quantitative morphometric analyses were conducted blind, using coded slides and micrographs, and all statistical analyses were performed by determining the mean value for each animal, and averaging these individual mean values to obtain the group mean. Thus, the statistical populations were equal to number of animals, for all statistical tests (analyses of variance).

Tissue preparation and sampling. The ultrastructural analyses conducted in this study were among the larger quantitative electron microscopic (EM) analyses in the field, and involved counting and measuring well over 5,000 synapses, performing point-counting analyses on nearly 1,000 micrographs, and measuring areas for hundreds of glial inclusions and glial profiles. However, the tissue sampled by our procedures still represented only a tiny fraction of total hippocampal neuropil. Thus, it was essential to use highly systematic and consistent procedures in preparing, sectioning, and analyzing the tissues. After fixation, we dissected out the hippocampus and cut transverse, 250-µm-thick slices from the middle of the septotemporal axis of the structure. Based on cell layer patterns visualized under a dissecting microscope, and on the septotemporal location, a consistently similar slice was selected from each animal for analysis. This slice was then dehydrated, stained, and embedded in Araldite according to standard EM procedures. The embedded tissue was trimmed on a plane perpendicular to the medial-lateral axis of the slice and parallel to the CA1 apical dendrites, on a line through CA1 and the dentate gyrus (using consistent landmarks of the pyramidal and granule cell layers). Semithin (1 µm) and thin sections (60 nm; silver-gray interference color, stained with uranyl acetate and lead citrate) were then cut from the block.

Electron micrographs were obtained from the apical dendritic region of CA1 (stratum radiatum), midway between the pyramidal cell somal layer (stratum pyramidale) and the fibers of the perforant path (stratum moleculare). This is the main terminal field for the Schaffer collateral-commissural pathway (e.g., Swanson et al. 1978). A series of 20 micrographs per subject was shot in a planned sequence across this region, at an initial magnification of 12,500 X. The micrographs were photographically enlarged to 32,000 X for analysis. Another five micrographs were shot of the five largest glial cell processes on the sections. Criteria for acceptable fixation quality were normal, unswollen mitochondria, sharp membranes with little intercellular space, well-dispersed synaptic vesicles within the synaptic terminals, and normal-appearing myelin. Tissues not meeting these criteria were excluded from the analysis.

Light microscopy. The nucleoli of pyramidal cells were counted across the stratum pyramidale on at least five semithin sections per animal. Only neurons that were clearly within the pyramidal somal layer were counted. The width of this cell layer on each section was measured with a calibrated eyepiece, and data were expressed as neurons/100 µm stratum pyramidale length.

Nucleolar diameters and length of stratum radiatum, i.e., length of apical dendrites, also were measured for each animal, in order to ensure (1) that nucleolar diameter was not changed by the treatment (since larger diameter structures are more likely to be counted) (cf. Weibel 1979), and (2) that changes in overall structure size (which can affect neuronal density) had not occurred.

Electron microscopy. Synaptic active zones were defined as dark paramembranous densities associated with at least two synaptic vesicles. The total number of "edge synapses," i.e., synapses at the edge of a micrograph, was divided by 2 before being included in the count, to avoid overestimation of synaptic density (since edge synapses theoretically appear in more than one micrograph).

Each active zone length was measured, using a digitizing pad and morphometric software and hardware (Bioquant), and was simultaneously coded for the type of terminal with which it was associated. Terminals were categorized as simple axospinous, perforated, axodendritic, or multiple spine (cf. Peters et al. 1976). Multiple spine synapses are characterized by two spines in contact with one terminal. Mean active zone length was calculated separately for each category for each animal.

The number of synapses per unit volume [N_v] can be estimated from the number of synapses per area [N_a] of micrograph, using stereological correction factors. That is, if synapses are significantly larger than a fraction of the section thickness, they will theoretically often be present in two or more sections, and will, therefore, be overestimated. To correct for this, the average length of synapses in micrographs (d) is incorporated with section thickness (t) into the denominator of the formula for converting N_a to N_V (Weibel 1979):

$$N_v = \frac{N_a}{d + t}$$

Since each individual synaptic category was analyzed separately (which substantially reduces variability in trace length), the coefficients of variation for individual categories were well below 20 percent (cf. Weibel 1979). and the separate synaptic categories were, therefore, treated as monodispersed populations in our analyses. The total N_v was determined by adding the N_V's of the individual categories together.

Estimates of relative volume for each element of the neuropil were determined using a 10 x 12 grid overlay on each micrograph. The area immediately under each intersection of the grid was examined and categorized for the element of neuropil it contained. Neuropil elements were classified as terminal, axon, dendritic spine, dendritic shaft, glial process, or unknown.

The volume fraction of each element in the neuropil was determined for each subject from the fraction of test points [P_p] that were

classified as a given element of neuropil. That is. the fraction of test points [P_p] that cover a given element on a two-dimensional representation is an unbiased estimator of the fractional volume [V_v] of that element (Weibel 1979), expressed as:

$$P_p = V_v$$

Mean volumes (v) for terminal and spine elements were readily calculated by combining the above measures, i.e., dividing the volume fractions [V_v] by the appropriate N_V for each element:

$$v = \frac{V_v}{N_v}$$

Neuroanatomical Results

A significant decrease in hippocampal pyramidal cell density was found in the high-dose THC animals. The effect could be seen readily in some high-dose animals even without quantitative analysis. In several other high-dose animals, however, neuronal counts were within the range of the control group. Studies in which plasma hormones and THC are correlated with brain anatomic changes in the same animals may help to clarify the bases of these individual differences.

Quantitative EM analyses revealed no major changes in synaptic ultrastructure in THC-treated animals, despite the decrease in neuronal density. However, a tendency for mean lengths to be larger was seen in several categories for the high-dose group. In addition, a nearly significant tendency toward more multiple spine synapses was seen in high-dose THC-treated animals.

It has previously been found that multiple spines are more prevalent in the hippocampus during the synaptic reinnervation that follows a lesion (McWilliams and Lynch 1984; Steward and Vinsant 1983), perhaps reflecting remodelling or axon-sprouting phenomena. Conceivably, such compensatory activity could obscure changes caused by a gradual and mild loss of neurons induced by THC. Subsequent studies with higher doses of THC might accentuate or clarify this effect, and could therefore yield important clues to the nature of synaptic plasticity in the adult brain.

Although THC treatment did not substantially affect synaptic density, it did alter EM measures of astrocyte reactivity. Dark, membranous inclusions were present frequently in hippocampal astrocyte cytoplasm in THC animals, and a greater fraction of the cytoplasm of astrocytes was occupied by such inclusions in THC-treated animals. Similar inclusions, vacuoles. and globules increase substantially during normal aging (Landfield et al. 1981b; Vaughan and Peters 1974; Wisniewski and Terry 1973) or following experimentally induced brain lesions (e.g., Cook and Wisniewski 1973; Mugnaini et al. 1976).

CONCLUSIONS AND FUTURE QUESTIONS

The results of the long-term studies indicate that chronic exposure of young-mature subjects (of a mammalian species) to moderate doses of delta-9-THC is associated with reduced neuronal density and increased glial reactivity in the hippocampus. The higher dose level (8 mg/kg) was compatible with near-normal function on an active-avoidance task and near-normal food/water intake, and therefore does not seem to induce extreme sedation or toxicity, Despite the decline in neuronal density, synaptic density was not found to be decreased by THC, although a nonsignificant trend toward an increase in multiple spine contacts, i.e., two spines per terminal, was observed. This observation may reflect synaptic remodelling, sprouting, or compensation for terminal loss.

It is not clear why we did not observe an overall decline in hippocampal synaptic density in THC-treated rats, similar to that described in a recent abstract by Scallet et al. (1986). Further studies may pinpoint the methodological or rat strain factors that account for these differences.

Many aspects of blood chemistry were normal in our THC-treated rats, although cholesterol, glucose, and possibly uric acid and creatinine were elevated, while plasma protein and albumin were decreased. Some of these effects also have been observed in other studies (e.g., Rosenkrantz et al. 1975). In addition, "stress" hormones such as ACTH and corticosterone were elevated in our THC-treated rats. Interestingly, many of these same blood chemistry and hormonal changes have been found in aged rats, as noted above.

The glial and neuronal density changes are extremely similar to those which we have previously found to occur during normal aging in rats. We have shown that these changes can be retarded or prevented by chronic adrenalectomy (Landfield et al. 1979; Landfield et al. 1981a). Therefore, of particular interest in this regard was the observation that the THC treatment resulted in an elevation of plasma ACTH and corticosterone.

One hypothesis that might account for these findings is that THC may bind to steroid receptors, mimicking the effect of glucocorticoids (e.g., Rawitch et al. 1977; Solomon et al. 1976; Szara 1973), thereby accelerating brain aging. An alternative possibility is that THC might increase ACTH release, possibly by interfering with negative feedback inhibition in the adrenal-pituitary axis (Drew and Slagel 1973), or by more direct effects, thereby elevating endogenous corticosterone levels. In turn, higher corticosterone could induce the brain changes.

However, if one or the other of the above hypotheses is correct, then it is not obvious why synaptic density was not reduced with THC, or why no residual effects on maze-reversal learning were found, since both effects are seen (albeit subtly) in aged rats. One possibility is that, because the highest dose of THC was not particularly high in effective terms, the young animals in this

study were able to compensate for brain changes by learning and/or an axon-sprouting-dependent increase in multiple synapses. That is, young rats may be able to compensate more effectively for THC-induced cell loss than aged rats are able to compensate for age-related cell loss, perhaps because of an age-related decline in adaptive capacity. Thus, cell loss in young animals might result in morphological or behavioral patterns that differ from those caused by cell loss in aged animals, even if the cell loss results from relatively similar cellular mechanisms.

As a corollary to this view, it may be that rats chronically exposed to THC during early life will enter the later stages of life, e.g., over 20 months of age, with their brains closer to a "threshold" of pathology than will same-age animals not previously exposed to THC. Consequently, brain aging may be substantially more severe in THC-exposed rats than in same-age controls (due to either additive or synergistic effects between THC exposure and aging mechanisms), even if the animals have been withdrawn from THC for prolonged periods. Alternatively, some aspects of THC effects may be gradually reversible over prolonged periods, or, at least, normal aging processes may cause brain pathology in non-exposed animals to "catch up" to the pathology in THC-exposed animals.

In summary, there is increasing but not yet conclusive evidence that chronic THC exposure may interact with glucocorticoid systems and increase the rate at which aginglike anatomical changes develop in the brain. However, the morphological effects of THC on the brain appear to be subtle and gradual, and testing the various hypotheses regarding the mechanisms of these changes will likely require extensive and painstaking quantitative analyses in animals treated for prolonged periods.

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Mechanisms of Tolerance to Opiates in Locus Coeruleus Neurons

Macdonald J. Christie, John T. Williams, and R. Alan North

INTRODUCTION

The phenomena of tolerance and physical dependence resulting from the repeated administration of opioids have been observed in a variety of experimental models. Despite a considerable research effort, however, the underlying molecular mechanisms remain to be described. Difficulties in identifying appropriate biochemical processes have arisen predominantly from a lack of experimental models for the study of tolerance and dependence in isolation. Thus, studies of opioid tolerance are often confounded by the presence of associated physical dependence, the presence of multiple opioid receptor subtypes, or the measurement of a biological effect which is either remote from the opioid-sensitive neuron or is rendered grossly unphysiological by experimental manipulations. Intracellular recording from neurons of the locus coeruleus (LC) in vitro provides a method that avoids these difficulties and thereby offers a useful experimental approach to the mechanisms underlying opioid tolerance.

MECHANISMS OF ACUTE OPIOID ACTIONS ON LC NEURONS

An inherent advantage of the study of opioid actions using intracellular recordings of membrane potential or ionic conductance from single neurons is that the measured physiological response is in close temporal and spatial proximity to opioid receptor activation. The effects of opioids on single LC neurons are illustrated in figure 1. Intracellular recordings were made as described in detail elsewhere (North and Williams 1985). A thin slice of rat pons containing the nucleus was superfused with a solution similar in composition to cerebrospinal fluid. When the opioid agonist Met-enkephalin (ME) was added to this solution, the spontaneous firing of the neuron was inhibited and the membrane hyperpolarized in a concentration-dependent manner (figure 1A). Figure 1B illustrates membrane currents induced by ME recorded under voltage clamp. Such measurements of membrane current allow direct calculation of membrane conductance, which represents the proportion of open ionic channels. Using these methods, it has been shown that

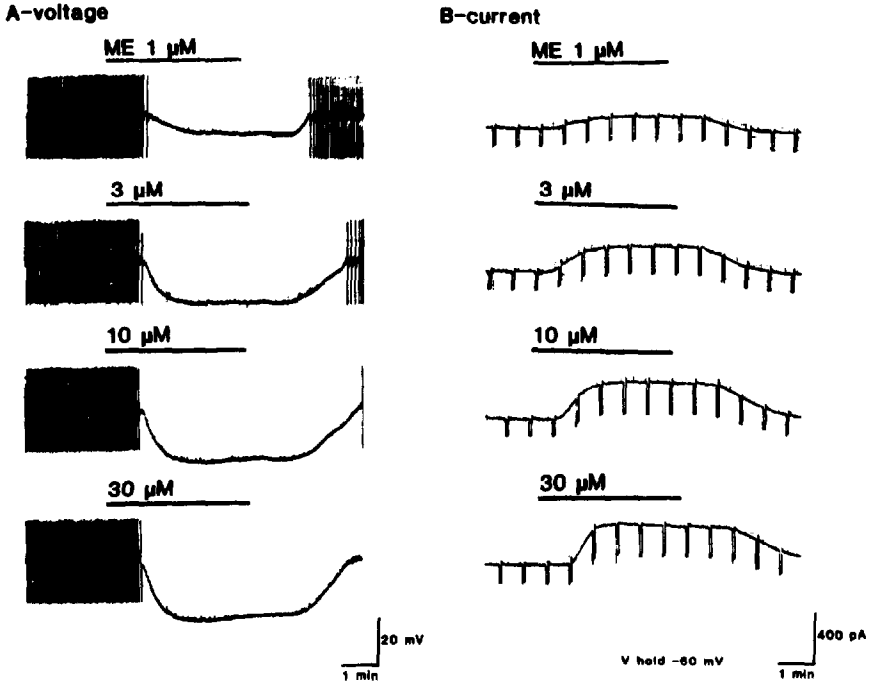

FIGURE 1. *Intracellular recordings of the action of ME on single LC neurons*

NOTE: (A) This shows that the tiring of a spontaneously active LC neuron is inhibited and the membrane Is hyperpolarized when ME is present In the solution that superfuses the brain slice. Upward deflections are action potentials (full amplitude not shown), and downward deflections are after hyperpolarizations. (B) Recording of membrane current from a rat LC neuron voltage clamped at -60 mV. The four traces show the membrane currents caused by various concentrations of ME (outward current Is upward). Downward deflections on these records are the currents flowing in response to repeated application of a fixed hyperpolarizing pulse. The increase in amplitude of the current during the presence of ME indicates that the membrane conductance is increased.

opioids produce a hyperpolarization of LC neurons by increasing their membrane conductance to potassium ions (Williams et al. 1982; North and Williams 1985).

A further advantage of this procedure for the investigation of opioid tolerance is that opioids increase potassium conductance in LC neurons by interacting with a single class of opioid receptors. This conclusion is largely based on the use of selective opioid

antagonists to characterize receptors by the method of Arunlakshana and Schild (1959). Thus, the dissociation equilibrium constant (K_e) of naloxone, derived from Schild analysis, using a variety of opioid agonists (ME, [D-Ala^2 ,D-Leu^5]-enkephalin (DADLE), normorphine, [D-Ala^2,MePhe^4,Gly^5-ol]-enkephalin (DAGO)) was 2 to 3 nM, indicative of a v-receptor (Williams and Narth 1984; Williams and North, unpublished observations). The K_e of the δ-selective antagonist bis-alyl-tyr-aib-aib-phe-leu (ICI 174864) for receptors on LC neurons was 6 μM, which is much larger than the value obtained for tissues containing only' δ-receptors and is also consistent with the presence of μ-receptors.. Furthermore, the highly selective δ-agonist [D-Pen^2,D-pen^5]-enkephalin and the selective K-agonist trans-(+-)3,4-dichloro-N-methyl-[2-(1-pyrrolidinyl)cyclohexyl] benzeneacetamide methane sulfonate (U50488H) were ineffective in inhibiting LC neurons and increasing potassium conductance at concentrations as high as 10 μM (Williams and North 1984; Williams and North, unpublished observations).

The mechanism of coupling between u-receptors and potassium channels has not been directly demonstrated in LC neurons, but several lines of evidence strongly suggest that a pertussis toxin-sensitive guanine nucleotide-binding protein (G-protein) is involved (although inhibition of adenylate cyclase is not). u-Agonist binding to brain membrane preparations is sensitive to guanosine-triphosphate (GTP) and magnesium (Chang et al. 1981). suggesting a G-protein interaction , and treatment of rats with pertussis toxin has been shown to eliminate opioid-induced hyperpolarization of LC neurons (Aghajanian and Wang 1986). Finally, although there is evidence that opioid receptors are negatively coupled to adenylate cyclase in various systems (West and Miller 1983). the hyperpolarization or outward current caused by ME was not influenced by the adenylate cyclase activator forskolin (North and Williams 1985). or by the metabolically stable analogs of cyclic adenosine 3',5'-monophosphate (cAMP) 8-bromo-CAMP and dibutyryl-CAMP (Williams and North, unpublished observations), suggesting that changes in intracellular levels of cAMP are not involved in the coupling of u-receptors to potassium channels. A report of inhibition of opioid hyperpolarizations of LC neurons by cAMP analogs (Andrade and Aghajanian 1985) was subsequently shown to be the result of the activation of an inward current by these compounds (Wang and Aghajanian 1986).

α_2-Adrenoceptors on LC neurons also appear to be coupled via a pertussis toxin-sensitive G-protein to the same set of potassium channels as those activated by opioids in LC neurons. Like hyperpolarizations induced by opioids, those caused by α_2 adrenoceptor agonists were also abolished by pretreatment of animals with pertussis toxin (Wang and Aghajanian 1986). and α_2-adrenoceptor agonists increased a potassium conductance which had the same properties and the same maximum value as that caused by opioids (North and Williams 1985; Williams et al. 1985). Experiments in which an opioid and an α_2-agonist were superfused together indicated that the same potassium conductance is increased by both

agonists. This permits localization of any adaptive processes following chronic opioid treatment either to mechanisms common to μ- and α_2-receptors, or selectively to μ-receptors (figure 2).

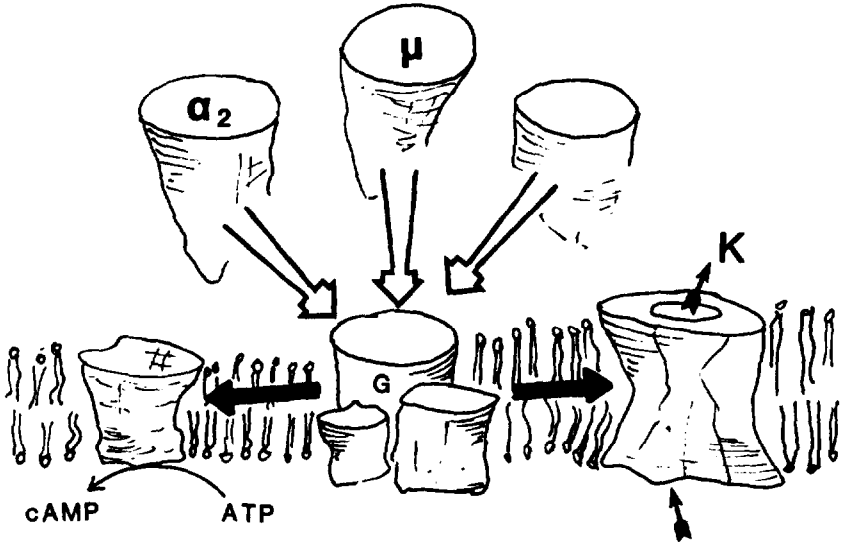

FIGURE 2. *Model of the mechanism of action of μ- and α_2-agonists on LC neurons (see text for details)*

NOTE: Both μ- and α_2-receptors (and perhaps other receptors) open potassium channels. This action involves a G-proteln. Although activation of these receptors can also be shown under some circumstances to inhibit adenylate cyclase, this seems not to be Involved in the opening of potassium channels.

These studies have largely resolved the mechanisms involved in the primary physiological action of opioids on LC neurons. The aim of the experiments described below was to use similar procedures to record membrane currents from LC neurons (isolated from morphine-tolerant animals) to study the changes in the properties of μ-receptors, potassium channels, and the coupling between these elements that occur as a consequence of long-term morphine administration.

TOLERANCE BUT NOT DEPENDENCE FOLLOWING CHRONIC MORPHINE TREATMENT

Concentration-response relationships to DAGO and normorphine demonstrated the development of tolerance to opioids following chronic treatment of rats with morphine (Christie et al., in

press). Animals were implanted with a total of 16 morphine pellets (75 mg base) over 7 days. Although the maximal outward current induced by DAGO was not significantly affected, the EC50 was shifted to the right twofold as a result of chronic morphine treatment. In control tissue, the maximum outward current induced by normorphine (30 μM) was similar to that induced by DAGO. The maximum response evoked by normorphine in tissue from morphine-treated rats, by contrast, was only about one-half of that produced by DAGO. and the EC50 was shifted to the right twofold. Therefore, the degree of tolerance to DAGO was about twofold throughout the concentration-response curve, whereas the degree of tolerance to normorphine ranged from about sixfold at the foot of the concentration-response curve to infinite at about one-half of the initial maximum response. These results were not a consequence of the presence of residual morphine in the brain slice, because the observed tolerance persisted for up to 6 hours after removal of tissue from the animal and could not be mimicked by preincubation of the slice in morphine (10 μM, 1 hour).

No manifestations of physical dependence were observed in individual LC neurons following chronic morphine treatment. In the absence of opioid agonists, naloxone was without effect on membrane current, or the frequency of spontaneous action potentials in either control tissue or tissue from morphine-treated rats. In the presence of opioids, naloxone (1 μM) simply reversed the membrane hyperpolarization or outward current, even when the superfusion of the agonist had been continued for up to 4 hours (figure 3). That is to say, at the level of the individual neuron, naloxone did not produce any effect on the properties of the cell other than a simple reversal of the ongoing action of morphine, whether or not the neuron was removed from a rat which had been chronically treated with morphine. These results are consistent with extracellular recordings from LC slice preparations (Andrade et al. 1983). One set of in vivo experiments originally suggested a modest increase in firing rate of LC neurons following systemic or iontophoretic application of naloxone in chronically treated animals (Aghajanian 1978). This excitation probably results from a reversal of the action of circulating morphine and/or from an increase in the activity of excitatory afferents to LC during in vivo experiments. A dissociation of opioid tolerance from physical dependence has also been reported in preparations of the mouse vas deferens (Muster et al. 1982).

ELIMINATION OF SPARE RECEPTORS FOLLOWING CHRONIC MORPHINE TREATMENT?

The observation that tolerance to normorphine was more pronounced than that to DAGO (Christie et al., in press) might appear anomalous if both agonists were interacting with the same type of opioid receptors on LC neurons. However, such a discrepancy would be expected if chronic morphine treatment resulted in a loss of the number of opioid receptors on the neuronal surface, because in this case the degree of tolerance to a particular agonist would

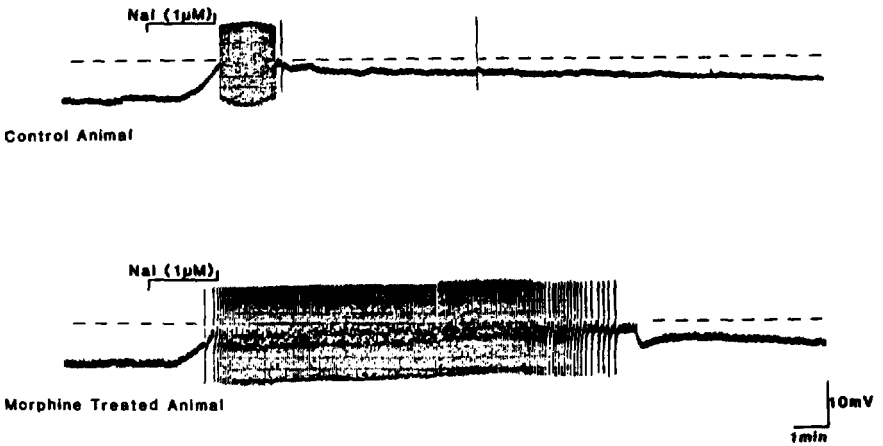

FIGURE 3. *The effect of naloxone on the membrane properties of two LC neurons*

KEY: Top trace: Record from a cell removed from a naive rat. Bottom trace: Record from a cell taken from a rat which had been chronically treated with morphine.

NOTE: Slices from control and treated rats were superfused with morphine for 3 to 4 hours prior to addition of naloxone (Nal) to the superfusion solution. Naloxone produced a simple reversal of the hyperpolarizing action of morphine in both cells. The longer period of firing in the lower trace presumably results from the delayed onset of the action of morphine as the naloxone washes out as a result of the tolerance to morphine.

depend on the fraction of receptors which that agonist must normally occupy to produce a given effect. For instance, if normorphine is a partial agonist, i.e., it must occupy a large fraction of the receptors on the cell surface in order to produce a maximal increase in potassium conductance, and DAGO is a full agonist, i.e., it can produce a maximal effect on potassium conductance while occupying only a small fraction of the receptors on the neuron, then the action of normorphine would be more sensitive than the action of DAGO to the loss of receptors. Such an effect of chronic morphine treatment would be formally analogous to the

acute elimination of receptors by treatment with an irreversible antagonist, which is often done to measure spare receptor populations (receptor reserve) and to estimate the affinity constant of agonists (Stephenson 1956; Furchgott and Bursztyn 1967).

Several lines of evidence suggest that the effect of chronic morphine treatment is very similar to that of acute irreversible antagonism--reduction of spare receptors or receptor reserve. First, full activation of potassium channels in normal LC neurons occurs with low fractional occupancy of u-receptors by full agonists (Williams and North 1984). Second, normorphine must occupy a greater fraction of p-receptors to elicit an equivalent response to that produced by DAGO, i.e., it possesses lower efficacy (Miller et al. 1986). Thus, in control tissue, both normorphine and DAGO are capable of eliciting the same maximal response, since there is considerable receptor reserve. In treated tissue, normorphine is not capable of eliciting the same maximal response as DAGO because fewer recognition sites are present. An alternative explanation, presented below. is that the coupling of p-receptors to potassium channels has become less effective.

This interpretation was further tested by irreversible antagonism of a fraction of the u-receptor population with ß-chlornaltrexamine (CNA, 30 nM for 30 minutes). If chronic morphine treatment eliminates spare u-receptors, then the actions of both normorphine and DAGO should be more sensitive to CNA in neurons from animals made tolerant to morphine. This was found to be the case (Christie et al., in press). The maximal outward current induced by both DAGO and normorphine was reduced to a greater extent by CNA in tissue from chronically morphine-treated rats than in control preparations. The K_e of naloxone for the µ-receptors was unchanged. These results are consistent with the suggestion that there are fewer µ-receptors available on LC neurons from rats chronically treated with morphine.

Elimination of spare receptors does not necessarily imply a reduction of the number of opioid recognition sites on the surface of the neuron, but could also be due to a less efficient coupling between u-receptors and potassium channels. If we assume that elimination of surface recognition sites is solely responsible, i.e., chronic morphine treatment is equivalent to partial irreversible antagonism, then Furchgott analysis (Furchgott and Bursztyn 1967) predicts that a 70 to 80 percent reduction in U-receptor density would be required to produce the observed shift and reduction in the maximum of the normorphine concentration-response curve. This possibility seems unlikely because opioid binding studies have generally failed to observe any reduction in the density of binding sites following chronic morphine treatment (Creese and Sibley 1981). Thus, the reduction in u-receptor reserve following chronic treatment appears to be due to a postreceptor adaptation.

Chavkin and Goldstein (1984) and Porecca and Burks (1983) arrived at similar conclusions using the method of partial irreversible antagonism in the morphine-tolerant guinea pig ileum preparation.

Because several processes intervene between opioid receptor occupation and tissue response (muscle contraction) in that preparation, no conclusions could be drawn concerning the nature of the postreceptor adaptation. Thus, it was not clear if the response decrement was associated specifically with μ-receptors or opioid-sensitive neurons, or if it was a more general tissue response.

OPIOID-INDUCED POTASSIUM CONDUCTANCE IS UNCHANGED FOLLOWING CHRONIC MORPHINE TREATMENT

The membrane properties of LC neurons and the increase in conductance induced by maximal concentrations of DAGO were unaffected by chronic morphine treatment, suggesting that the population of potassium channels activated by opioids was not modified by chronic morphine treatment. Spontaneous action potentials had amplitudes close to 80 mV, rose from a "threshold" potential of -55 mV, and had durations of about 1.5 ms; these properties were the same in neurons from both groups of rats. Under voltage clamp, neurons from both groups had the same conductances and showed the same types of rectification (Williams et al. 1984).

The increase in conductance induced by maximal concentrations of DAGO (3 μM) or ME (30 μM) was determined by constructing steady state current-voltage relationships in the absence and presence of agonist and then subtracting the resting conductance from that found in the presence of agonist over the same potential range. The increase in conductance induced by DAGO or ME was not significantly affected by chronic morphine treatment (Christie et al., in press). These results demonstrate that DAGO and ME were able to activate fully the potassium conductance of LC neurons following chronic morphine treatment and that the mechanism underlying tolerance in LC neurons is confined to processes prior to the mechanism by which full p-receptor agonists activate potassium channels.

α_2-ADRENOCEPTOR ACTIONS FOLLOWING CHRONIC MORPHINE TREATMENT

Chronic morphine treatment did not change the effects of agonists acting at α_2-adrenoceptors on LC neurons. The outward currents induced by maximal concentrations of the full α_2-agonists noradrenaline (10 μM) and 5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline (UK14304, 1 μM) were similar to those induced by DAGO and ME and were not significantly affected by chronic treatment (Christie et al., in press). The outward current induced by a maximal concentration of clonidine was consistently less than that induced by noradrenaline or UK14304 and was also not affected by chronic morphine treatment. Because clonidine is incapable of fully activating the α_2-receptor-mediated increase in potassium conductance despite full receptor occupation, i.e., clonidine is a partial agonist, it should be particularly sensitive to any reduction in coupling efficiency. Therefore, it can be concluded that the mechanisms underlying tolerance to morphine are specific to μ-receptors and/or their coupling to potassium channels, rather than involving any mechanism common to both μ- and α_2-receptors.

NALOXONE DISSOCIATION CONSTANT FOLLOWING CHRONIC MORPHINE TREATMENT

Chronic morphine treatment was without effect on the K_e of naloxone at the -receptors. Concentration-response relationships for DAGO or ME were constructed in the presence of increasing concentrations of naloxone in both control and treated tissue, and the naloxone pA_2 was determined by Schild analysis (Arunlakshana and Schild 1959). It was similar in both groups, giving a K_e of 2 nM (Christie et al., in press). This finding is supported by radioligand binding studies which have also shown no change in antagonist binding K_e following chronic morphine treatment (Creese and Sibley 1981). Several pharmacological studies which utilized Schild analysis observed changes in naloxone affinity in vivo and in vitro (Tallarida et al. 1979). but these studies generally failed to account for the presence of residual morphine in the tissue, the presence of multiple opioid receptor subtypes, or signs of physical dependence in the preparations under investigation.

CONCLUSIONS

In conclusion, the present results demonstrate that chronic treatment of rats with morphine caused opioid tolerance but no signs indicative of physical dependence in individual neurons of the rat LC. The tolerance was the result of selective elimination of spare μ-receptors. There was no effect on the naloxone K_e at the μ-receptor, and there were no changes in the effects of agonists at α_2-adrenoceptors or in the properties of the potassium conductance which couples to both of these receptors. Although this specific elimination of spare μ-receptors could be the consequence of a reduced density of μ recognition sites on each neuron, available evidence indicates that it is more likely due to a specific reduction in the efficiency of coupling of u-receptors to potassium channels.

The reduction in the efficiency of coupling of μ-receptors to potassium channels following chronic morphine treatment is confined to mechanisms prior to those common to both μ- and α_2-receptors. Because both μ- and α_2-receptors appear to couple to potassium channels via a pertussis toxin-sensitive G-protein (see above), one might speculate that the most likely mechanism of tolerance is the reduction in the ability of μ-receptors to couple to the G-protein. Such a mechanism has a parallel in the mechanism of homologous desensitization of δ-receptors in NG 108-15 cells. In that system, although receptor internalization occurs, it is preceded by a homologous desensitization of agonist-induced inhibition of adenylate cyclase (Law et al. 1983). which correlates with a reduced ability of δ-agonists to stimulate the GTPase activity of a pertussis toxin-sensitive G-protein (Vachon et al. 1985). Similarly, homologous desensitization of ß-adrenoceptor-stimulated adenylate cyclase in rat lung membranes precedes receptor internalization and appears to be due to sequestration of recognition sites into microdomains of the plasma membrane which

are inaccessible to adenylate cyclase (Strasser et al. 1985). a process involving receptor phosphorylation (Sibley et al. 1985). Similar mechanisms could account for the reduced coupling of μ-receptors to potassium channels in LC neurons following chronic morphine treatment.

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Cholinergic Modulation of Sensory Responses in Cerebral Cortex

John P. Donoghue

INTRODUCTION

The cerebral neocortex receives input from a number of systems that are thought to act as modulators of cortical activity. These systems are characterized anatomically by their widespread input to the cortex and, where they have been studied, are associated functionally with sleep, arousal, vigilance, and more generalized activities. Widespread systems contrast with specific thalamocortical input systems, such as the geniculostriate visual pathway, which have highly localized axonal arbors in cortex and are associated with the transmission of detailed place- and modality-specific information. While the neurotransmitters of the specific systems are still unknown, those for at least four widespread input systems have been established: the norepinephrine (NE) input from the locus coeruleus, the dopaminergic input from the mesencephalon, the serotonergic input from the raphe nuclei, and the cholinergic (ACh) input from the basal forebrain area. The identification of transmitters for these systems has made it possible to examine their role as modulators of cortical function by combining pharmacological, behavioral, and electrophysiological techniques.

This chapter will focus on the function of the cholinergic system in cerebral cortex. This system has received considerable attention because it has been repeatedly linked to processes involved in memory,learning, and synaptic modification in cerebral neocortex.Fibers from the basal forebrain region provide the bulk of the cortical cholinergic input, although there also appear to be a small number of cholinergic neurons within the cortex itself, at least in rodents (Jordan and Phillis 1972; Johnston et al. 1981; Wenk et al. 1980; Lehmann et al. 1980). While basal forebrain fibers have a widespread distribution to the entire cerebral cortex, they exhibit a rough topography in their cortical projection (Kievit and Kuypers 1975; Divac 1975; Mesulam and Van Hoesen 1976; McKinney et al. 1983; Saper and Loewy 1980; Lamour et al. 1982a).These features of the basal forebrain input suggest that single cholinergic neurons influence large cortical areas, but the

system as a whole may be functionally and regionally segregated. Lesions or drug manipulations that interfere wfth the normal action of ACh alter the acquisition, storage, or retrieval of information as measured in a variety of behavioral tasks in man and other mammals (Davis et al. 1978; Deutsch 1971; Drachman 1977; Friedman et al. 1983). ACh has also been implicated along with norepinephrine in controlling the ability of experience to modify cortical sensory response properties during development (Bear and Singer 1986). Together, these results suggest that the mechanisms for selective processing and storage of information in cortex are closely tied to the cellular actions of ACh in cortex.

In this chapter, I will discuss the role of ACh as a modulator of cortical cell excitability based on early electrophysiological analyses of the effect of ACh on cortical spontaneous activity, then I will present results of more recent experiments that take a different approach to examine cholinergic function in cerebral neocortex. These investigations show how the naturally evoked discharge of neurons in the somatic sensory cortex is altered by local iontophoretic application of ACh.

CHOLINERGIC EFFECTS ON THE DISCHARGE OF CORTICAL NEURDNS

General Effects on Background Activity

One common means to assay the function of a putative neurotransmitter is to test the effect of a local iontophoretic application of that transmitter and related compounds on the background discharge of a neuron. This procedure has been used extensively to study cholinergic action in neocortex. Iontophoretic application of ACh reportedly excites or inhibits the background discharge of individual cortical neurons (Krnjevic and Phillis 1963a; Krnjevic and Phillis 1963b; Krnjevic 1971; Krnjevic et al. 1971; Spehlmann and Smathers 1974; Vazquez et al. 1969; Randic et al. 1964; Phillis and York 1967). More recent studfes have emphasized the predominantly excitatory actions of ACh (Lamour et al. 1982b; Lamour et al. 1983) that appear to be a consequence of a decrease in two or more specific membrane K^+ conductances (Krnjevic et al. 1971; Woody et al. 1978; McCormick and Prince 1985). Despite the important contribution to our understanding of the pharmacological actions of ACh in cortex from studies of background modulation, it has been difficult to establish the role of ACh in cortical information processing, learning, memory, or other complex cortical processes with this type of analysis.

Modulation of Sensory Responses by ACh

Another strategy has evolved to examine the potential modulatory role for various widespread cortical inputs. Instead of examining change in rate of background activity as a measure of drug action, these studies have focused on the way modulators alter the pattern of response or receptive field characteristics of cortical neurons. This form of analysis has as its goal an understanding

of the role of modulatory substances in cortical information processing. In a series of seminal studies on transmitter action in cerebral cortex, Krnjevic (Krnjevfc and Phillis 1961; Krnjevic and Phillis 1963a; Krnjevic and Phillis 1963b) used local iontophoretic glutamate application to test the ability of ACh to modify cell excitability. Doses of ACh that were insufficient to alter spontaneous discharge rate nevertheless enhanced the excitatory response of a cortical neuron to glutamate application (Krnjevic and Phillis 1963b). Since cortical afferent fiber systems, such as the specific thalamocortical system, are thought to use transmitters that act in a manner similar to glutamate, these results suggest an important modulatory role for ACh: the presence of ACh could enhance the response of cortical neurons to sensory inputs. This form of modulation of synaptic strength could form a basis for selective attention, intracortical filtering, or, if synaptic strength changes were to persist, could provide one mechanism for storing information.

Recent studies in my laboratory (Donoghue and Carroll 1987) have employed the strategy of combining iontophoresis with single unit recording to examine the form and distribution of cholinergic modulation of sensory response properties of neurons located in the whisker representation of the rat somatic sensory cortex (SI). The highly ordered and modular organization of this system makes it an attractive model for studies of functional organization in cortex. In the rat, each whisker has a distinct representation in SI cortex that can be visualized in tissue sections as aggregates of granule cells in layer IV, each termed a "barrel" (Woolsey and Van der Loos 1970). Each cell within this layer IV barrel receives its dominant sensory input from a single whisker, and the actual pattern in which barrels are arranged in the cortex matches the pattern of whisker follicles on the rat's face (Welker 1971; Welker 1976; Simons and Woolsey 1979). We have examined cholinergic modulation of sensory responses in the SI barrel field because, using this system, it is possible to study the relationship between a discrete cortical territory and its peripheral end organ, because it is easy to deliver a controlled natural stimuli to the whiskers in the cell's receptive field, and because functional properties of cells in this area are being studied intensively (Ito 1981; Simons 1978; Simons 1983; Simons 1985).

In our experiments, we are using the SI-whisker system as a model to identify the way modulatory systems alter sensory processing in the cortex. Specifically, we are examining four aspects of cholinergic modulatory action:

(1) How does ACh modify sensory responses of SI neurons?

(2) What receptor type mediates these effects?

(3) Are these effects localized to specific subsets of cortical neurons?

(4) Does ACh produce a long-term change in sensory response?

In the following sections, I will present results of some experiments that address the first three questions. Issues related to the temporal aspects of ACh function (the fourth question) are addressed in the chapter by Dykes (this volume).

Method of Testing ACh Modulation of Sensory Discharge

Our experiments were carried out in urethane-anesthetized (1.8 g/kg IP) adult albino rats. Glass, multibarrel electrodes were used to record single unit activity and to apply drugs iontophoretically. Drug barrels were filled with ACh chloride (0.2 M, pH 4), atropine sulfate (0.1 M. pH 5), GABA (0.1 M, pH 4) or glutamate (0.1 M, pH 8), and 165 mM NaCl. After the whisker that yielded a maximal discharge of the unit under study had been identified, precisely controlled air puffs were delivered to the whisker to quantify its -activity. Each isolated cell was tested during whisker stimulation under three basic conditions: (a) with no applied drug, (b) during local application of ACh (mean ejection current 34±22 nA, range 5 to 100 nA), and (c) after a 3- or 5-minute recovery period. In some cases, atropine was coapplied with ACh to test for the specificity of ACh action. The laminar location of each recorded cell was determined from histological reconstructions of electrode tracts in thionin-stained sections through the recording area and by electrophysiological features of recorded neurons.

Features of Response Modification During ACh Application

During ACh application, either enhancement or suppression of the discharge evoked by whisker stimulation could be observed in SI neurons. Cells were divided into two general types based on this difference: Type 1, which showed an increase in sensory-evoked discharge, and Type 2, which showed a decrease in discharge. Among the ACh-modulated SI neurons (n=68), Type 1 effects predominated (figure 1). Seventy-three percent of the ACh-affected cells increased their peak firing frequency when the sensory stimulus was delivered during ACh iontophoresis. This change in peak firing frequency reflected a change in the number of spikes elicited by each stimulus and, in some cells, also reflected an increase in the probability that sensory stimulation would evoke discharge. Type 1 cells were further subdivided into three groups based on differences in the pattern of discharge modulation by ACh, The distribution of these types is shown in figure 2. In the first type (1a), which accounted for most (75 percent) of the Type 1 cells, the overall discharge of the cell was increased by ACh (figure 3). Thus, both the mean rate of ongoing (background or-"spontaneous") discharge and the peak discharge evoked by whisker stimulation increased when ACh was applied. Type lb cells increased peak discharge during ACh application without change (<10 percent) in background discharge rate (figure 4). Thus, for these cells, which comprised 11.1 percent of all Type 1 cells, ACh enhanced the response evoked by sensory stimulation without

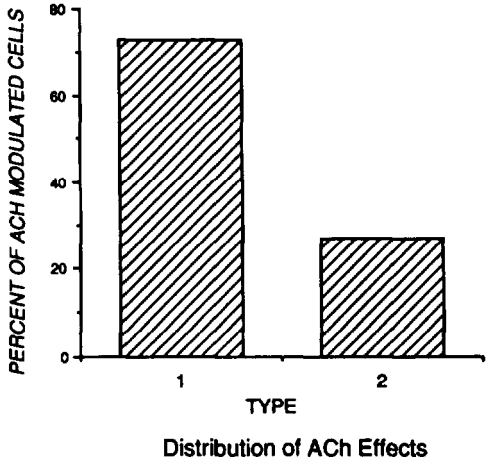

FIGURE 1. *Distribution of Type 1 (response-enhanced) and Type 2 (response-suppressed) cells*

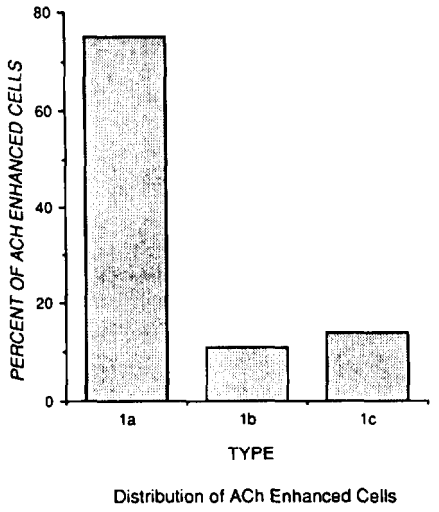

FIGURE 2. *Distribution of different forms of Type 1 cells*

NOTE: (1a) Increase in background and evoked discharge during ACh application. (1b) Increase in response without change in background discharge. (1c) No response to sensory stimulus unless ACh is applied.

affecting the cell's spontaneous discharge. Type 1c cells (13.9 percent of Type 1 cells) were difficult to find because they generally had low rates of spontaneous activity and did not respond to air puffs or mechanical stimulation at any peripheral location in the absence of ACh. Consequently, brief glutamate applications were used to evoke spontaneous activity and locate these cells.

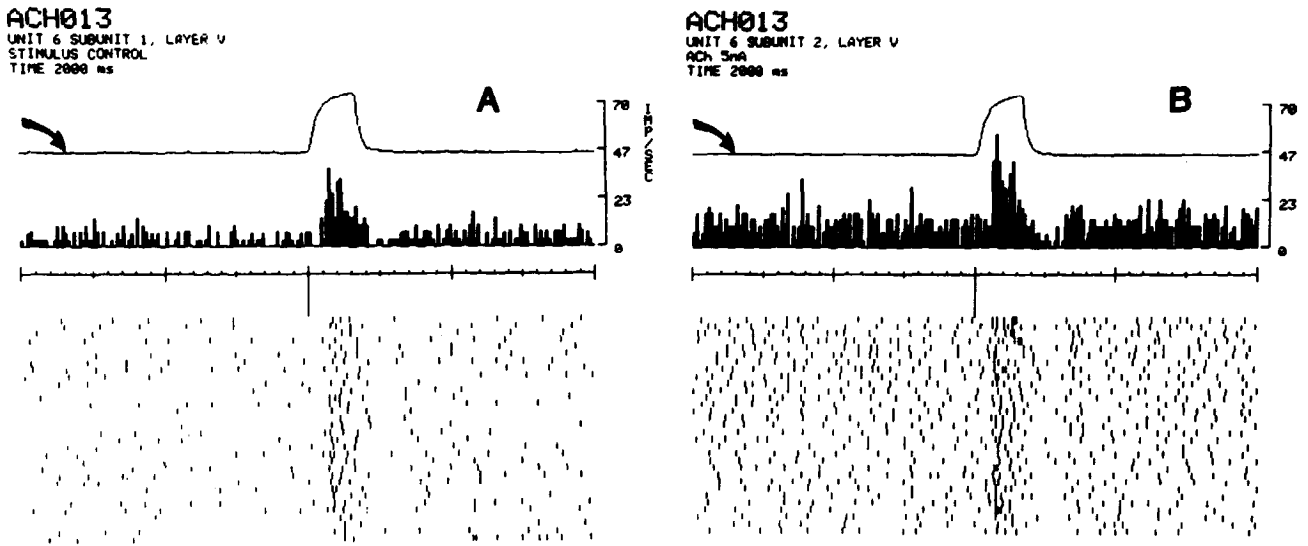

FIGURE 3. *Example of Type 1a cell*

NOTE: These displays show the response of a single layer V neuron before (A) and during (B) the application of 5 nA of Ach. Each rester (bottom) shows the spike discharge over a 2-second period for 30 presentations of an air puff to the whisker et the center of the cell's receptive field. Average spike frequency Is displayed in the histogram above (bin width 10 ms). The upper trace in each display (arrow) shows the time course end amount of air pressure delivered to the whiskers by the stimulator. Note the Increase In the amount of discharge evoked by the stimulus and In the level of background discharge during ACh applicatIon. The same general format is used In figures 4-6.

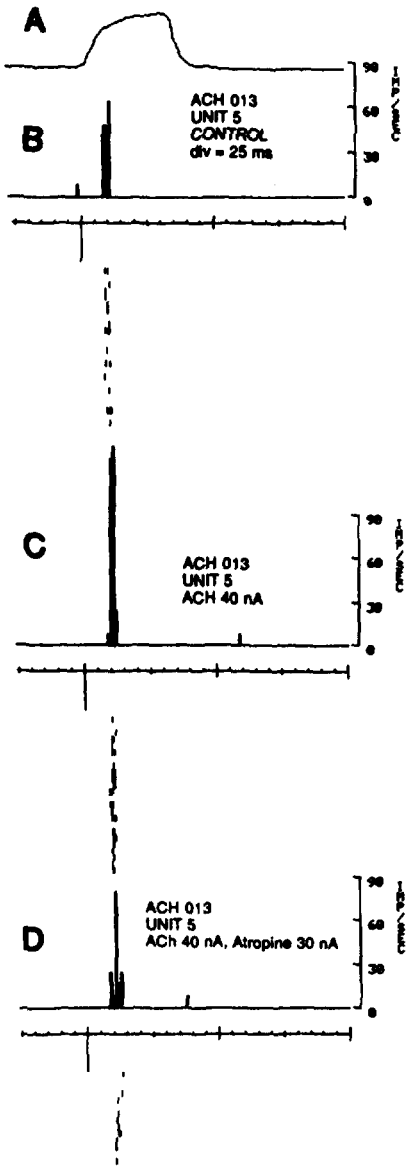

FIGURE 4. *Type 1b enhancement*

NOTE: (A) Analog signal of the air puff stimulus temporally aligned with histograms below. (B) Control histogram (no applied ACh). (C) Response during application of 40 nA of ACh. (D) Response during concurrent application of 40 nA ot ACh and 30 nA of atropine. Note the increase in response during ACh application without change In background discharge. The finding that 1 enhancement is blocked by atropine suggests that this effect is mediated via muscarinic receptors.

When a sensory stimulus was presented in combination with ACh iontophoresis (figure 5). these cells were activated by relatively small deflections of one or a few whiskers. The ACh-dependent receptive field of these cells was centered about the same whisker as nearby neurons that were driven by tactile stimulation in the absence of ACh. ACh had little effect on background discharge for most of these cells, but increased background discharge was observed in a few instances, as in the cell shown in figure 5. Unlike other Type 1 cells which show a simple increase in evoked activity, ACh appears to act like a gate in Type 1c cells: sensory-evoked activity depends on the presence of ACh.

The three subtypes of ACh enhancement described above may not represent three distinct categories. These subtypes may be members of a continuum whose responses appear different because they depend on the membrane potential of the neuron at the time of ACh application (Adams et al. 1982; Cole and Nicoll 1984b). Cells with relatively little ACh effect on background discharge might be hyperpolarized beyond the range at which a voltage-dependent ACh effect could be expressed. This explanation would clarify the previously described positive correlation between spontaneous discharge and degree of ACh effect (Jones and Olpe 1984). Peripheral stimulation could bring the cell into a range where ACh is effective in increasing excitability, thus accounting for an increase in evoked response without background modulation. The low ongoing activity of these cells is consistent with this explanation. Thus, the form of ACh modulation may reflect the particular membrane potential of a cell rather than effects mediated by separate receptor types or by different classes of cortical neurons.

Modulatory effects of ACh have also been observed in cat sensory cortex. Sillito and Kemp (1983) reported that 61 percent of neurons recorded in the visual cortex of anesthetized cats showed a larger response to visual stimuli in the presence of ACh, much like our Type la or lb effect. Recently, response enhancement has been reported in cat SI and auditory cortex as well (Metherate et al. 1985; McKenna et al. 1986; Dykes, this volume). These results in two widely separate species and in different sensory areas suggest that ACh has a similar functional role throughout mammalian cerebral neocortex.

ACh suppressed sensory responses (Type 2 effect) in 27 percent of all ACh-modulated cells in rat SI cortex (figures 1 and 6). The decrement in the peak response was generally small, averaging a decline of 24.6±20 percent compared to pre-ACh controls. One unusual finding was that 50 percent of Type 2 cells showed a paradoxical increase in background discharge that accompanied the sensory response decrease.

Previous studies have generally reported ACh-induced inhibition only in a small percentage of cells (Krnjevic et al. 1971; Randic et al. 1964; Lamour et al. 1982b; Lamour et al. 1983; but see Phillis and York 1967). However, these cells, identified by the depression of background discharge by ACh, are different from

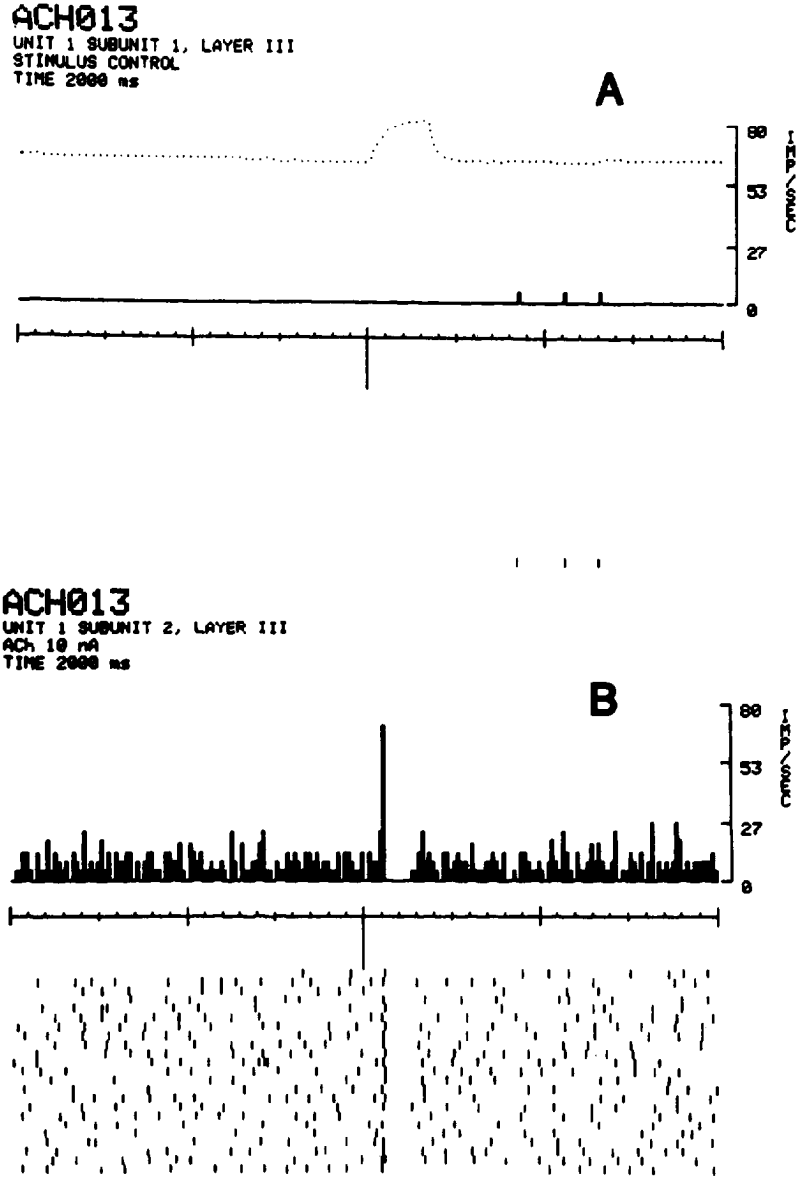

FIGURE 5. *Type 1c cell located in layer III*

NOTE: Characteristic of these cells, there Is no response to any somatic sensory stimulus (A). During ACh application, the response to whisker deflection is evident (B).

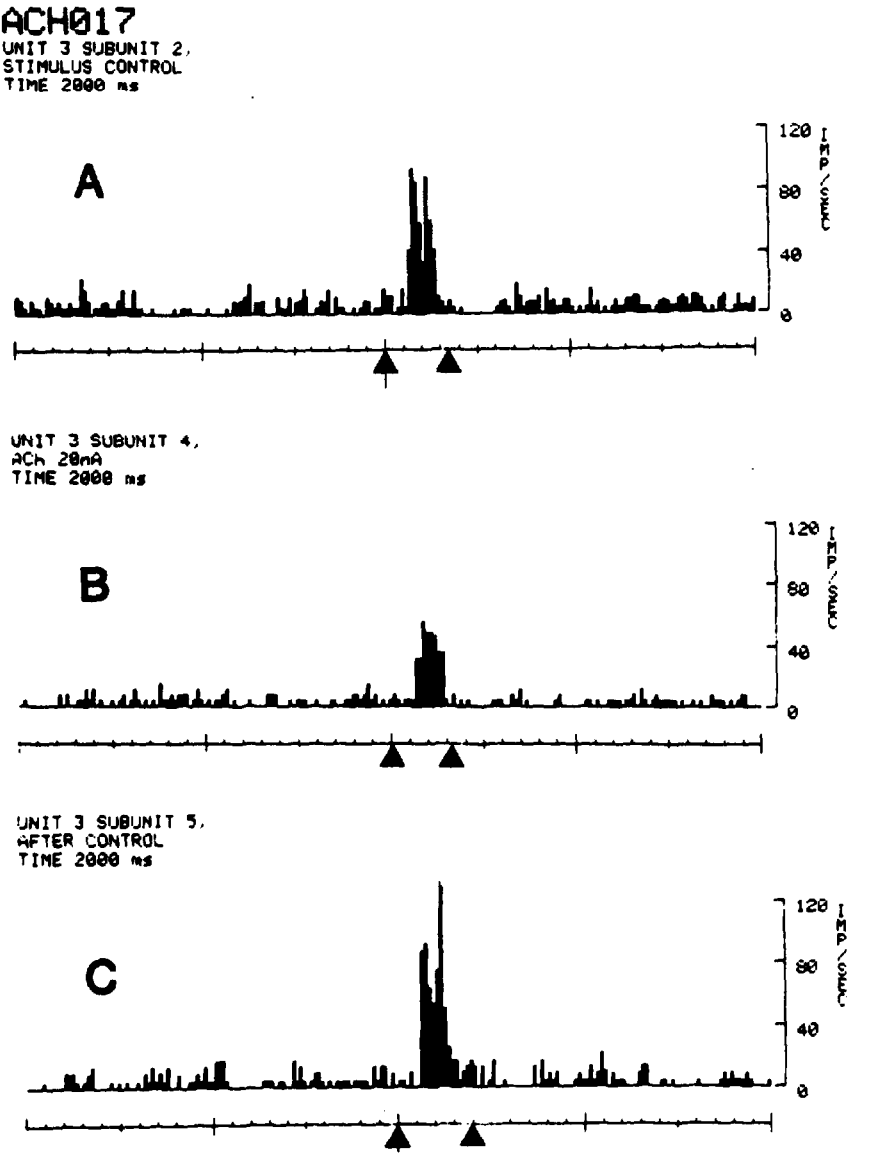

FIGURE 6. *Type 2 cell located in layer III*

NOTE: Stimulus was presented during the period marked by the two arrowheads. (A) Control. (B) During the application of 20 nA of ACh. (C) Response histogram collected beginning 3 minutes after the termination of the ACh application.

those presently described as Type 2, which show that suppression is largely, if not entirely, restricted to the discharge of the cell evoked by natural stimulation within the cell's receptive field. General background discharge suppression was not commonly found in rat SI. Type 2-like changes in visual responses were observed in 30 percent of the ACh-modulated cells in cat visual (VI) cortex (Sillito and Kemp 1983). Thus, similar percentages of cells appear to be suppressed by ACh in cat VI and rat SI cortex.

Amount of Response Modulation by ACh

The extent of response modulation by ACh was calculated as the $[(Peak_{ACh}-Peak_{control})/Peak_{control}X100\%]$, where $Peak_{ACh}$=the peak discharge amplitude (averaged over 20 trials) evoked by whisker stimulation during ACh application; $Peak_{control}$=peak discharge before ACh application. The average discharge increase for all Type 1 cells during ACh application was 72±11 percent, and changes as high as 278 percent were observed. Cells for which receptive fields could be found only after ACh application were not included in this sample. The percent increase in response averaged for all cells (enhanced and suppressed) is illustrated in figure 7.

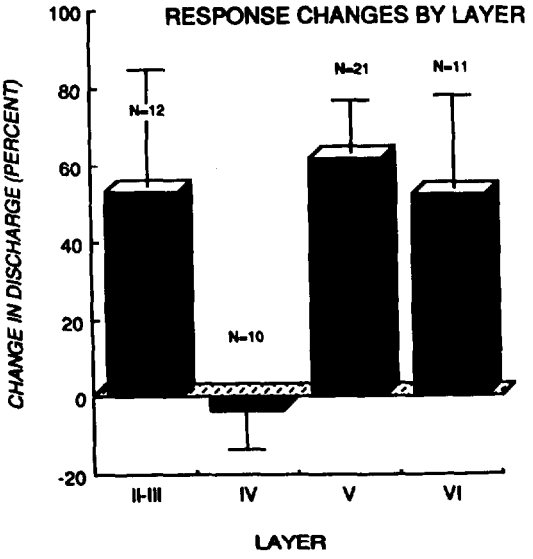

FIGURE 7. *Average response modulation by ACh shown by layer*

NOTE: This histogram shows the mean overall change In response (enhancement or suppression) by layer, expressed as a percent change In discharge. No cells were recorded in layer I. Cells In layers II-III were grouped together. Bars indicate one standard deviation of the mean. Note that ACh enhancement is found in supra- and infragranular layers and that layer IV shows the opposite effect of ACh application compared to all other layers.

The quantitation of peak discharge change may be a somewhat misleading measure of ACh effect, since we do not know whether the iontophoretically applied ACh in any way approximates the concentration of ACh that acts upon cortical ACh receptors. Dose-response tests (Lamour et al. 1982b) at least support the idea that modulation of background discharge is dependent on the amount of ACh delivered (as measured by iontophoretic current level). We have also observed in a few cells that the enhancement of peak discharge by ACh is related to the iontophoretic current level. It would be of great use to measure and compare the levels of ACh released by basal forebrain fibers and by iontophoretic electrodes or to devise methods to control ACh release from basal forebrain fibers.

Receptor Types

A number of previous studies have documented the predominantly muscarinic nature of ACh-induced increases in background activity (Krnjevic and Phillis 1963b; Krnjevic et al. 1981; Phillis and York 1967; Crawford and Curtis 1966; Stone 1972; Lamour et al. 1982b; Lamour et al. 1983; McCormick and Prince 1985). The site of muscarinic action, however, has been a matter of debate: both pre- and postsynaptic receptor locations have been suggested (Rovira et al. 1983; McCormick and Prince 1985). Nicotinic effects on cortical discharge are thought to be uncommon (Stone 1972; McLennan and Hicks 1978) and in some cases unrelated to ACh action (Lamour et al. 1982b). This action is discussed further in the following section on "Predominance of Type 2 effects on layer IV."

We tested the role of muscarinic receptors in controlling Type 1 and Type 2 modulation of sensory responses with the muscarinic antagonist atropine. Concurrent application (tested in 29 cells) of ACh and atropine either markedly diminished or abolished the increase in background and evoked discharge that was induced by ACh (figure 4). This effect was demonstrated for all forms of Type 1 and Type 2 cells. Thus, it appears that muscarinic receptors account for both response enhancement and response decrement. This suggests that there may be separate subtypes or distributions of receptors, or that receptors are linked to different ion channels or second messengers, to account for these differences.

Selective Distribution of Cholinergic Effects

Despite the broad distribution of ACh fibers in cortex, ACh effects may be restricted to specific classes of cortical neurons. Lamour and coworkers reported that neurons showing background discharge modulation by ACh in rat SI cortex were mainly localized to two infragranular bands: one in the deep part of layer V and the other in the lower part of layer VI (Lamour et al. 1982b; Lamour et al. 1983). Many of the ACh-responsive neurons in layer V were identified as pyramidal tract (PT) neurons (Stone 1972; Woody et al. 1974; Lamour et al. 1982b) which convey output from somatic sensorimotor cortex to the spinal cord and to brain stem sensory

relay and motor control structures. Other layer V and VI ACh-sensitive cells were identified as corticothalamic (CT) neurons, and more than half of all PT and CT neurons were excited by ACh (Lamour et al. 1982b). Lamour et al. (1982b) concluded that a second band of ACh-sensitive neurons in the deep part of layer VI were interneurons, based largely on their inability to activate them antidromically from the thalamus, pyramidal tract, or contralateral cortex. These studies, based on examination of the ability of ACh to alter background activity, suggested that ACh modulation is selective for cortical output neurons and one set of deep-lying local circuit neurons; the background activity of intrinsic and cortically projecting pyramidal neurons which reside in the superficial layers were relatively unaffected by ACh.

Laminar Distribution of ACh Effects in Rat SI Cortex

Recent studies that have examined the effect of ACh on sensory-evoked discharge have identified a much different, but nevertheless selective, distribution of ACh-sensitive neurons in cortex. Most noteworthy are the findings that enhancement is found in superficial as well as deep layers and that enhancement and suppression effects are segregated by layer.

Type 1 distribution. Our studies in the rat SI cortex showed that enhancement effects are present in all cortical layers. In layers II and III, 83 percent (10 of 12) of the cells tested increased their mean peak response following ACh application, whereas the sensory-evoked discharge increased for 91 percent (29 of 32) of layers V and VI cells. The remaining two superficial and three deep layer cells were Type 2; two Type 1 cells were present in layer IV.

Other recent reports confirm a broad laminar distribution of ACh-induced excitation in cortex. McCormick and Prince (1985) found increases in background discharge in more than 90 percent of the pyramidal cells studied in a population of 253 intracellularly recorded layers II and III neurons in rat cingulate cortex slices. Sillito and Kemp (1983) also found that ACh enhanced roughly similar numbers of neurons in superficial and deep layers of cat primary visual cortex. These recent reports provide strong evidence for an ACh-mediated modulation of excitability in superficial cortical layers that was missed when only background discharge effects were monitored.

Predominance of Type 2 effects on layer IV. Cells showing a suppression of evoked response were largely restricted to the superficial layers; only 9 percent of the ACh-responsive infragranular cells had Type 2 responses. This finding is consistent with earlier reports that ACh suppression of glutamate-evoked excitation was primarily observed in the superficial layers (Phillis and York 1967). In contrast to all other layers, Type 2 effects were the predominant response type in layer IV. Electrophysiologically, cells located in the region of layer IV (about 600 to 800 μm below the cortical surface) exhibit a distinctive set of

features: they are often more difficult to isolate than cells at other depths (due to their small size), they are extremely sensitive to low-amplitude deflections of a single whisker, and their receptive fields include one or, at most, a few whiskers (Simons 1978; Chapin and Lin 1984; Pincince and Donoghue 1986). Reconstructions of penetrations observed in thionin-stained sections through SI confirmed that 80 percent of the cells that showed response suppression were located in layer IV. Thus, the ratio of response-enhanced to response-suppressed (Type 1/Type 2) cells was reversed for layer IV compared to deeper or more superficial layers (figure 7). Sillito and Kemp (1983) also found that cortical cells inhibited by ACh were most common in layers III and IV and were absent in deep layers of cat visual cortex. Recently it has been reported that nicotinic receptors are densest in layer IV of cat visual cortex (Prusky and Cynader 1986). which suggests that nicotinic receptors may be located presynaptically on thalamocortical afferent fibers. Activation of this presynaptic receptor could explain our finding that ACh decreases sensory responses in layer IV of rat SI. However, this interpretation is not consistent with our finding that Type 2 effects can be blocked by atropine or that nicotinic receptors appear to be most dense in layer III of rhesus monkey cortex (O'Neill et al. 1985), although many thalamic afferents terminate in layer III in the monkey. Further studies with specific nicotinic agonists and antagonists are clearly required to identify the mechanism for Type 2 effects. It is curious that ACh suppresses evoked discharge in layer IV, the primary target for specific thalamocortical fibers, while it enhances responses in other layers.

Overall, studies of the effect of ACh on sensory-evoked discharge in cortex demonstrate a markedly broader distribution of cholinergic actions than was identified by studies of background modulation. Anatomical studies of the cholinergic input to cortex support this conclusion. Cholinergic fibers are most dense in layers I, V, and VI, based on choline acetyltransferase immunoreactivity (Chao et al. 1982), acetylcholinesterase staining (Mesulam and Van Hoesen 1976; Mesulam et al. 1983; Mesulam et al. 1984). and axonal transport studies (Rieck and Carey 1984). Pyramidal cells in all layers may be modulated by ACh release upon their apical dendrites that ascend to layer I, and pyramids in the deeper layers may also be influenced by ACh action on more proximal dendrites. In contrast to the pronounced effect of ACh in layer V and the presence of presynaptic cholinergic markers, most studies agree that muscarinic receptors are lowest in layer V (Nonaka and Moroji 1984; Rotter et al. 1979; Wamsley et al. 1980; O'Neill et al. 1985).

Segregation of Cholinergic Effects Among Different Cell Classes

In addition to a distinct laminar segregation of effect, ACh has distinctly different effects on intrinsic local circuit and pyramidal neurons. Using intracellular recording and dye injection in a cortical slice preparation, McCormick and Prince (1985) found that ACh application to multipolar, presumably GABA-inhibitory

neurons resulted in a rapid, relatively brief burst of action potentials. This activity resulted in an IPSP in adjacent pyramidal cells. Excitatory ACh effects on pyramidal neurons were slower in onset and longer in duration, and this type of response could be blocked somewhat selectively by pirenzipine, suggesting that different muscarinic receptor subtypes mediate these two quite diverse effects. ACh has also been reported to decrease inhibitory action in the hippocampus, perhaps by direct action on GABA terminals (Krnjevic et al. 1981; Ben-Ari et al. 1981). Each of these results suggests that ACh may shape sensory processing by regulating the effectiveness of local cortical inhibitory circuits.

CONCLUSIONS

The combination of sensory stimulation and afferent activation to study the effects of modulators has revealed features of cholinergic action that would not have been observed if effects on background discharge alone had been examined. Based on all available evidence, we hypothesize that a primary action of ACh in cortex is to enhance sensory responses of neurons in supragranular and infragranular layers. This effect may result from the "destabilizing" effect of ACh produced by blockade of an outward Ca^{++} dep$ndent K^{+} current that follows neuronal discharge and a second K^{+} current that resembles the M current (Benardo and Prince 1982; Cole and Nicoll 1984a; Cole and Nicoll 1984b; Halliwell and Adams 1982; Brown 1983; McCormick and Prince 1985). Presynaptic effects of ACh have also been suggested as a mechanism for cholinergic enhancement effects (Krnjevic et al. 1981; Valentino and Dingledine 1981). In layer IV, there is a reversal of effect so that sensory responses are suppressed in the presence of ACh. The net result of these actions on sensory responses in cortex may be a filtering out of the weakest inputs at the earliest stages of processing in layer IV and a relative increase in the processing of the remaining signal in supra- and infragranular layers. Sensory activation of some cortical neurons is dependent on ACh as shown by our Type 1c cells which responded to sensory inputs only in the presence of ACh.

It is important to take into consideration that these modulatory effects in rat SI cortex were described in anesthetized animals. In awake animals, these same cells may have somewhat different responses to ACh. However, state-dependent changes in sensory responsiveness have been identified in SI of awake, behaving rats (Chapin and Woodward 1981), and the basal forebrain cholinergic system could be one way that these gating effects are achieved. It will be critical to show that other means of providing ACh at cortical synapses, such as direct stimulation of cortically projecting basal forebrain neurons, produce the same types of effects as those that have been described here using local iontophoretic application techniques.

The cellular actions of ACh suggest that the basal forebrain system is not a completely generalized system. There appears to be

selective modulation of certain aspects of sensory signals and a selective laminar and cell class segregation of ACh effects. Thus, the type of processing of sensory information in cortex will be dramatically changed if the basal forebrain or other cholinergic inputs are also active. The connection of this form of neural modulation to selective attention, memory, and other processes is still unclear. One could hypothesize, however, that activity in the basal forebrain could selectively enhance certain features of a sensory input by altering the strengths of synaptic activation of subsets of cortical neurons and could then facilitate the storage of those features in cortex.

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Control of the Neuronal Receptive Field in Somatosensory Cortex

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INTRODUCTION

Classic Receptive Fields

One of the more informative techniques to study the effects of drugs on the behavior of single neurons is to observe their activity in relatively normal physiological conditions both before and after a drug is administered to the neuron under study. By delivering substances iontophoretically through multibarreled pipettes into the vicinity of the neuron being studied, one can monitor changes in neural activity while the cell performs an identifiable function in a neural circuit under experimental control.

This approach can be illustrated in the somatosensory cortex, where neurons can be excited in a measurable and reproducible way by stimuli applied to the cutaneous receptive field. The examples chosen concern manipulations of two endogenous neurotransmitters, GABA and acetylcholine, in normal and partially deafferented cortex. To appreciate the findings presented, the following sections are preceded by a review of some fundamental concepts related to sensory processing.

Some of the earliest electrophysiological records provided the first glimpse of the organization of the receptive field. Hartline (1938) saw the excitatory influence from the omatidium of a horseshoe crab reduced by simultaneous illumination of adjacent omatidia. From this beginning came the idea that sensory systems process information through contrast enhancement between an excitatory center and an inhibitory surround (Hartline et al. 1952). This concept was so powerful that it not only won its discoverers the Nobel Prize but continues to be useful today. The first description of the center-surround organization of the receptive field in the mammalian nervous system was provided by Kuffler (1953) when he recorded the excitatory center and inhibitory surround of ganglion cells in the cat retina. These observations were followed by reports of center-surround organization in the somatosensory system. Investigators (Mountcastle 1957;

Mountcastle and Powell 1959) reported this organization in cortical neurons and in subcortical relays (Mountcastle and Darian-Smith 1968; Mountcastle 1986). Although it has become a basic tenet of models for processing of sensory information, after the initial discovery, the interaction of excitatory and inhibitory influences on somatosensory neurons has seldom been studied.

The Modern View: Dynamic Receptive Fields

To this, a notion of relatively static, concentric (but antagonistic) influences that shape the afferent signal at each relay from the periphery to the cortex, we must add some of the more recently described dynamic properties of somatosensory receptive fields.

Although it was originally thought that the change of state between wakefulness and sleep was sufficient to cause dramatic changes in the receptive fields of cortical neurons (McKenna et al. 1982), at least in primary somatosensory cortex, this does not seem to occur. The responses of more than 80 percent of cortical neurons in alert animals seem to be influenced only slightly by changes in attention or arousal. Hyvarinen et al. (1980) reported a general increase in activity and an enhancement of responsiveness to somatic stimuli in 16 percent of 160 neurons studied during a task requiring the attention of behaving monkeys. Chapin and Woodward (1981) showed that strong arousal produced only a slight decrease in spontaneous discharge and a slight increase in responsiveness of most neurons studied in rat somatosensory cortex.

In contrast, motor activity produces much more dramatic changes in responsiveness of somatosensory neurons. Generally, the responsiveness of somatosensory neurons was strongly suppressed during both grooming and treadmill locomotion (Chapin and Woodward 1981). Of 108 cells tested, 89 showed at least a 10 percent decline in the evoked response during running. Subsequent work demonstrated that the suppression observed during walking was phase-locked to the movement, so that suppression began just before the foot contacted the floor and was removed later in the step cycle (Chapin and Woodward 1982a; Chapin and Woodward 1982b). This kind of modulation of the sensory input may explain why receptive field organization assessed from observations of behaving monkeys manipulating test objects appears to be more variable in location and size than the organization seen in anesthetized or passive animals (Iwamura et al. 1983; Iwamura et al. 1985a; Iwamura et al. 1985b). Thus, it appears that large changes in receptive field organization may occur in behaving animals, but they are attributable to factors other than wakefulness or sleep.

Another important variable is the cytoarchitectonic area in which the neuron is located. Area 3b has the most stable receptive fields. Receptive fields in the adjacent area 1 seem to be more labile than those in area 3b and have more convergent inputs (Iwamura et al. 1983). Areas 5 and 7, which are further along in

the processing sequence, are known to be even more dependent on the motivational and attentional states of the animal (Mountcastle et al. 1975; Robinson et al. 1978; Bushnell et al. 1981). This discussion will be limited principally to neurons in area 3b, where receptive fields are less labile but more is known about both their inputs and the factors controlling receptive field organization. In this apparently less labile region of cortex, changes occur that can be related to neuronal plasticity and even to learning.

In examining changes that can occur in receptive fields, it is instructive to see how they change when a region of cortex loses its sensory inputs. Deafferentation induces rapid and dramatic changes in the somatotopic organization of sensory cortex in mammals (Kalaska and Pomeranz 1979; Rasmusson 1982; Kelahan et al. 1981; Wall and Cusick 1984; Merzenich et al. 1983a). These changes are progressive and reach new stable states only after a few months (Merzenich et al. 1983b). Studies of single neurons in deafferented regions suggest that, following deafferentation, many neurons acquire novel receptive fields; that neurons in many zones of the deafferented cortex remain responsive to cutaneous inputs (Kalaska and Pomeranz 1979); and that the proportion of neurons driven by cutaneous inputs attains the same level as in normal cortex (Dykes and Lamour, in press(a)). In monkeys (Merzenich et al. 1983a) and raccoons (Rasmusson and Turnbull 1983). the shift in receptive field locus following deafferentation begins to occur within hours or even minutes. If these changes occur through mechanisms comparable to those producing changes in receptive field loci in the spinal cord, then a novel receptive field can appear within seconds following loss of the afferent signal that provided the original receptive field (Metzler and Marks 1979). This argues strongly that sprouting of new terminals should be ruled out as a mechanism to explain the appearance of at least the first of the new receptive fields. Another point important for the following discussion is that the first receptive fields observed following deafferentation are not necessarily the final ones (Merzenich et al. 1983b), nor do the neuronal response properties immediately after deafferentation necessarily reflect the responses the neurons will have several days later (Rasmussen and Turnbull 1983).

BICUCULLINE

From the preceding discussion, it seems reasonable to infer that the receptive field of a somatosensory cortical neuron consists of some balance of excitation and inhibition, having a preferred spatial distribution but organized so that the relative strengths of the two processes can change rapidly in ways related to the behavior of the animal. These influences from nonsensory inputs seem to distort the preferred receptive field observed when the animal is at rest or unconscious. In the cortex, the predominant inhibitory neurotransmitter appears to be GABA. Existing histochemical evidence suggests that nearly all cortical interneurons contain this substance (Jones, in press), which plays an important role in

the control of receptive field size. As a consequence, blocking the inhibitory action of GABA shifts the balance between excitation and inhibition dramatically towards excitation.

In a series of experiments in cat somatosensory cortex (Hicks and Dykes 1983; Dykes et al. 1984), bicuculline (a GABA receptor blocker) was administered iontophoretically, and changes in neuronal behavior were recorded. Bicuculline had four different effects: (1) It increased the magnitude of the neuronal response elicited by stimulation of the cutaneous receptive field; (2) shortened the latency of the first action potential elicited by somatic stimuli; (3) enlarged the cutaneous receptive field; and (4) uncovered receptive fields in previously silent cells. These effects are summarized in figure 1.

The intensity of the response elicited from a cortical neuron was near maximum in the presence of bicuculline even when activated by very small somatic stimuli, such as the movement of a few hairs on the forearm, suggesting that GABA plays a role in controlling the amplification of the afferent signal within the cortex. Normally, stimuli applied near the edge of a receptive field produce weak responses (Mountcastle and Powell 1959; Gardener and Costanzo 1980), whereas the same stimuli applied to the center of the field produce larger and longer lasting responses. After bicuculline, large, long-lasting responses could be elicited by small stimuli on the edge of the receptive field, suggesting that the normal gain-control of the cell had been lost. The shortened latency of the responses (figure 1) observed in the presence of bicuculline suggests that GABA-mediated inhibition delays considerably the onset of the first action potentials in cortical neurons. The delay may be as much as 2 or 3 milliseconds, providing an opportunity for intracortical information processing to occur before a neuron discharges. This point has been amplified elsewhere (Dykes et al. 1988; Landry et al., submitted). Intracellular records have shown that sufficient time elapsed between the arrival of the thalamocortical input and the first spike to permit significant interactions among cortically generated excitatory postsynaptic potentials and inhibitory postsynaptic potentials.

The most striking change produced by bicuculline was an increase in the receptive field diameter of cortical neurons. In the presence of bicuculline, the size of the receptive field increased an average of 6.4 times the area of the control receptive field (Dykes et al. 1984). This average value masked some important differences in the degree of enlargement seen in different cells. Some receptive fields increased little more than twice their original size, whereas others increased as much as 20 times. Final receptive field size after enlargement was relatively constant, and size increases seemed to be related to the layer in which the receptive field was located. Figure 2 shows the original size of the receptive field expressed as a percentage of the receptive field observed after treatment with bicuculline. It can be seen that the control receptive field was less than 16 percent of the expanded field in the upper and lower layers but as much as 35

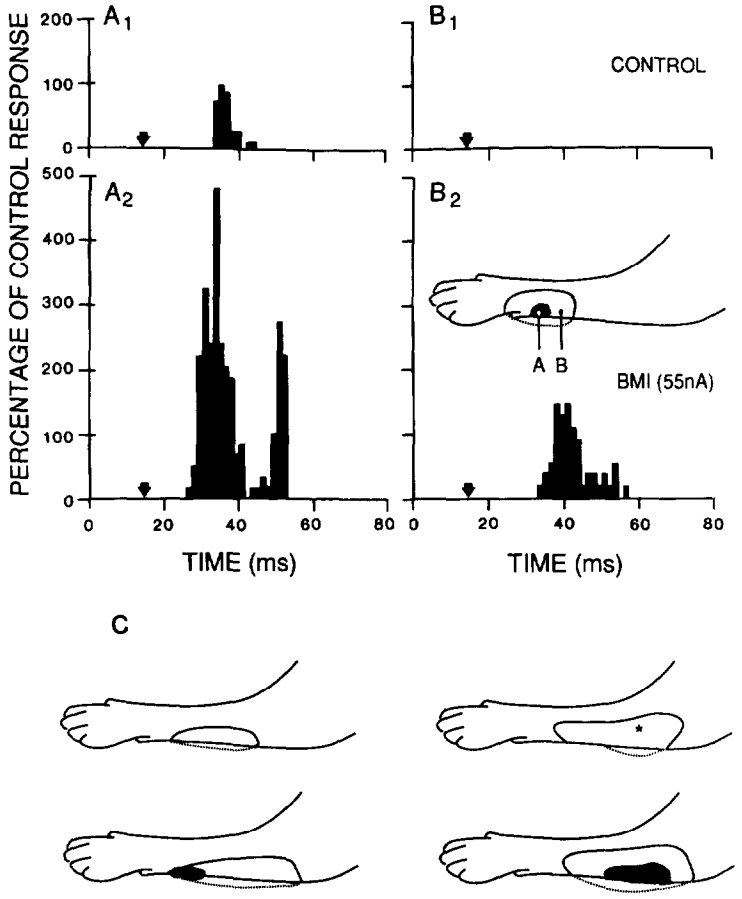

FIGURE 1. *Effects of biculculline administered iontophoretically to single neuron in cat somatosensory cortex*

NOTE: (A1) A histogram of responses to 10 stlmuli applied to the center of a cutaneous receptive field indicated as polnt A on the inset of the cat's forearm. (A2) A histogram of responses to 10 stimuli applied to the same point during bicuculline administratlon. (B1) No responses to 10 stimuli applied at polnt B outside the control receptive field on the cat's forearm, and (B2) a histogram of responses to 10 stimuli applied at point B during bicuculline adminilstratilon. Notice that the receptive field becomes enlarged during bicuculline admlnlstratlon. In addition, the response latency is shortened, and the magnitude and duration of the response are Increased. (C) Illustrations of other receptive fields affected by bicuculline administration. Some cells, having no (C, top left) or very hlgh (C, top right) threshold receptive fields, were converted to low threshold receptive fields, where movements of a few hairs could discharge the cells. Other previously existing receptive fields were enlarged (C, bottom left and right).

SOURCE: Modified from Dykes et al. (1984), Copyright 1984, the American Physiological Society.

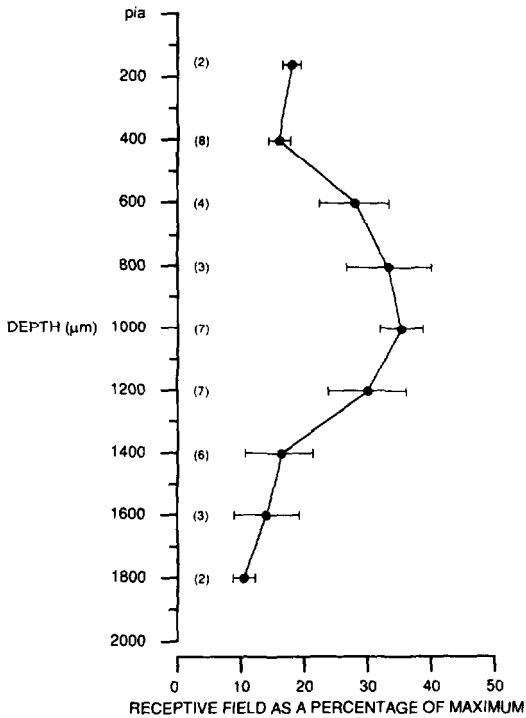

FIGURE 2. *Receptive field size as a function of cortical depth before bicuculline administration*

NOTE: The values plotted are those for the control receptive field expressed as a percentage of the area of the enlarged receptive field observed during GABA blockade by bicuculline. The greater enlargements observed In the upper and lower layers are attributable to a greater release of neurons In these layers from the effects of GABA-mediated inhibition. Numbers In parentheses indicate the number of receptive fields contributing to each mean and its standard error.

SOURCE: Replotted from Dykes et al. (1984), Copyright 1984, the American Physiological Society.

percent of the enlarged field in middle layers. The variable degree to which the receptive field increased appears to be due to the fact that GABA-mediated inhibition was more important in the upper and lower layers than in the middle ones.

It should be noted that bicuculline produces receptive field enlargements only in regions of cortex receiving inputs from rapidly adapting receptors. In regions that receive slowly adapting inputs, the responses to somatic stimuli are enhanced and their latencies decreased by administration of bicuculline, but the receptive field size remains unchanged. This implies that another

inhibitory neurotransmitter may control receptive field site or that receptive field size in slowly adapting areas is controlled by GABA through receptors insensitive to bicuculline. An effort to test the first hypothesis (Tremblay et al. 1986) has ruled out glycine, taurine, and ß-alanine as the unknown mediator of the inhibitory control. The lack of an effective GABA-A receptor blocker makes the second hypothesis difficult to test.

The data presented above provide no information about the dynamics of receptive field control. However, they show that, under normal circumstances, the observed receptive field is only a small part of the total excitatory drive on a cortical neuron, and they demonstrate that the major part of the excitatory convergence on a cell can be suppressed by intracortical inhibitory processes. Thus, factors that modify the inhibitory drive on a cortical neuron have the potential to alter the receptive field significantly. Similarly, if the excitatory drive could be enhanced sufficiently to overcome inhibitory influences, the receptive field would be changed. Thus, the rapid changes in receptive field organization which occur during an animal's behavior must arise from rapid, transient changes in this balance.

The last characteristic of somatosensory neurons important for the understanding of the dynamic properties of somatosensory cortex is that a large number of neurons (between 40 and 70 percent of those encountered in somatosensory cortex (Dykes et al. 1984; Dykes and Lamour, in press(b); see also Baker et al. 1971; Simons 1978) have no obvious receptive fields under most experimental conditions. However, many neurons without receptive fields developed them in the presence of bicuculline. Unresponsive neurons are most readily studied by administering glutamate through iontophoretic pipettes to show that a cell is present at the recording electrode and is capable of being discharged, even when somatic stimuli and/or thalamic electrical stimuli are unable to excite the neuron. By using iontophoretically administered glutamate to raise the level of excitability in the silent neurons or bicuculline to remove inhibition from them, it was possible to demonstrate that many silent neurons received excitatory inputs.

ACETYLCHOLINE

The large number of unresponsive neurons raises the question of what useful function such a neuron might have in somatosensory cortex. Some of the possibilities have been discussed by Dykes et al. (1984), Dykes and Metherate (in press), and Lamour and Dykes (in press). Here it is useful to discuss how these less than optimally excited neurons might play a role in two different circumstances where the cortex is known to undergo changes in receptive field organization. One such circumstance is that of directed attention, the other is deafferentation.

It is well known that conditions affecting alertness and attention, such as those involving the performance of a specific task for a reward or to avoid punishment, will double or triple the

amount of acetylcholine (ACh) released in the cerebral cortex (Rasmusson 1975). It was clearly demonstrated some years ago that iontophoretically administered ACh can cause 20 to 30 percent of cortical neurons to discharge (Krnjevic and Phillis 1963; Krnjevic 1975) and that many neurons have cholinergic binding sites, predominantly of the muscarinic type. In the visual cortex, Sillito (1977; Sillito 1979) showed that iontophoretic applications of ACh could alter the response properties of neurons so that their receptive fields were sharpened or changed in ways that enhance the signal-to-noise ratio of the response. In the cat somatosensory cortex, about 16 percent of the neurons (n=203) are excited by ACh (Metherate et al., in press), and in the rat, 37 percent (n=360) of the somatosensory cortical neurons are Similarly excited (Lamour et al. 1982; Lamour et al., submitted). These values encompass the range reported in earlier work on other cortical regions.

When the effects of somatic stimuli were studied during iontophoretic administration of ACh to cortical neurons, several interesting phenomena were observed. First, the responsiveness of a neuron to a somatic stimulus could be enhanced; thus, a larger response was generated to a fixed-amplitude stimulus, and the receptive field was defined more readily. In some cases, the threshold was lowered by iontophoretically administered ACh.

Second, during ACh iontophoresis, neurons previously unresponsive to somatic stimuli began to respond. Thus, ACh, like bicuculline, uncovered receptive fields in previously unresponsive neurons. The previously unresponsive cells had receptive fields of normal dimensions with appropriate locations for the region of cortex studied. Figure 3 illustrates some of the receptive fields uncovered in both cat (Metherate et al., in press) and rat (Lamour and Dykes, in press) somatosensory cortex. The uncovered receptor fields were approximately the same as those seen in neurons normally having receptor fields, with respect to size, thresholds, and response properties. In this way, iontophoretically administered ACh differed from iontophoretically administered bicuculline. Although bicuculline also uncovered receptive fields in previously silent neurons (Dykes et al. 1984; Dykes and Lamour, in press(b)), the receptive fields uncovered by bicuculline were abnormally large, and even very weak stimuli produced paroxysmal discharges in the neurons. In contrast, those uncovered by ACh had relatively normal sizes and response properties.

Hypothetically, the release of ACh which accompanies attention and directed effort enhances the responsiveness of most somatosensory neurons, making them better able to encode arriving sensory messages, and brings into action silent neurons that otherwise would be unresponsive to somatic inputs.

DEAFFERENTATION

The effects described above disappear soon after ACh administration stops. Previously silent neurons become unresponsive again;

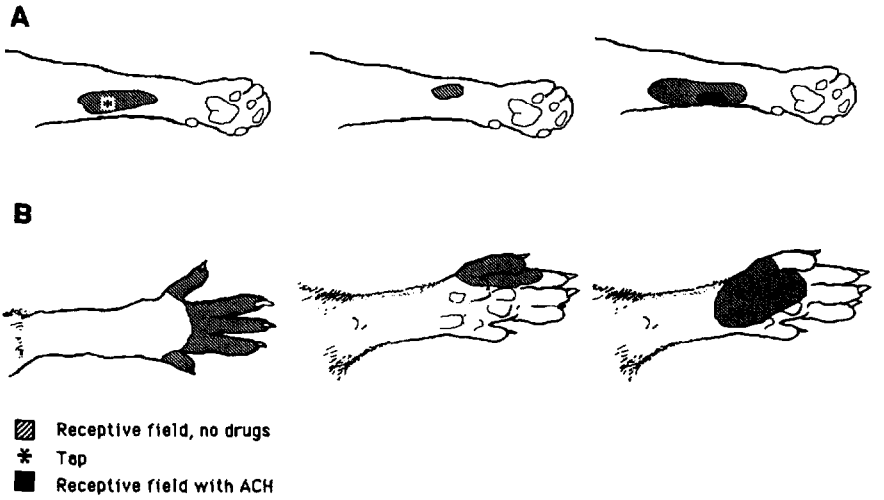

FIGURE 3. *Receptive fields of previously unresponsive neurons*

NOTE: Iontophoretic administration of ACh to (A) single cells In the primary somatosensory cortex of the cat and (B) neurons in rat somatosensory cortex uncovered low-threshold cutaneous Inputs capable of activating the cells.

SOURCE: Modified from Metherate et al. (in press), Copyright 1987, Pergamon Press; and Lamour and Dykes (in press), Copyright 1987, Elsevier Science Publishers B.V.

other neurons begin to generate fewer impulses and their thresholds often become elevated, returning to control levels. Such transitions may be comparable, in certain respects, to the changes that occur when attention is selectively shifted from one sensory modality to another or when motor actions involve a particular sensory surface. However, these kinds of reversible changes in excitability are insufficient to account for the reorganization seen in somatosensory cortex after a major deafferentation. After such a loss, some new responses are immediately unmasked, but the full sequence of changes following deafferentation often takes days to develop, following a slower time course and appearing to be relatively permanent.

Significant changes seen after deafferentation are:

(1) Neurons become less inhibited: They often discharge at higher than normal spontaneous rates and are depolarized more frequently by glutamate (Dykes and Lamour, in press(a); Lamour and Dykes, in press). They often respond with a rebound discharge to the release from inhibition (Rasmusson and Turnbull 1983).

(2) Neurons acquire new receptive fields in cutaneous regions adjacent to the region of deafferentation (Merzenich et al. 1983a; Wall and Cusick 1984).

(3) The somatotopic order which includes these new receptive fields becomes a new and apparently stable order within about 3 months (Merzenich et al. 1983b). Thus, relatively permanent changes occur in neuronal response properties related to receptive field organization.

ACh may also be involved in these relatively long-lasting changes in excitability of neurons in somatosensory cortex. It has been known for some time that ACh reduces potassium permeability in neurons (Krnjevic and Phillis 1963), and when ACh administration is combined with depolarization of the cell, to the point where the cell is discharged, the reduction of potassium permeability becomes relatively long lasting (Woody et al. 1978). In some cases, it may last for as long as the electrode can be kept inside the cell (up to 1.5 hours). This phenomenon occurs in neurons of cat motor cortex during the learning of a conditioned reflex, thereby enhancing the excitability of the neurons involved in the reflex (Woody and Engel 1972; Brons and Woody 1980).

To determine whether or not this effect has characteristics useful for explaining the reorganization of somatosensory cortex following deafferentation, the long-lasting effects of ACh were studied in cat cortex. The first experiments were designed to reproduce, in somatosensory cortex, the phenomenon described by Woody et al. (1978). Neurons were depolarized by iontophoretically applied glutanate, and ACh was administered. In 61 percent of 41 cells, this treatment produced enhanced responses to subsequent glutamate applications. This effect lasted for more than 5 minutes in 36 percent, and, in some, the enhanced responsiveness lasted for more than 45 minutes. In contrast, when the neuron was treated either with glutamate alone or ACh alone, very seldom was a prolonged change in excitability observed (figure 4).

The next step was to examine the effects of ACh on the responsiveness of neurons to somatic stimuli (figure 5). The excitatory glutamate test pulses were replaced by mechanical pulses applied to the cat's skin surface. In a series of 47 neurons having cutaneous receptive fields, 79 percent showed enhancement of the neural response, and 30 percent of these enhancements lasted for periods ranging from 8 minutes to 1 hour, and often for as long as the cell was studied. Thus, when ACh was combined with excitatory afferent drive, it caused long-term enhancement of neuronal excitability. Neurons originally poorly driven from the periphery developed well-defined cutaneous receptive fields, and light tactile stimuli within those fields effectively discharged them. In contrast, the excitability of a neuron was seldom changed by either somatic stimulation alone or ACh alone. In addition to the long-term enhancement of previously existing receptive fields, ACh treatment, when combined with neuronal excitation, uncovered receptive fields in previously silent neurons that, once expressed, remained for a long time. These appeared relatively normal in size and had normal response properties.

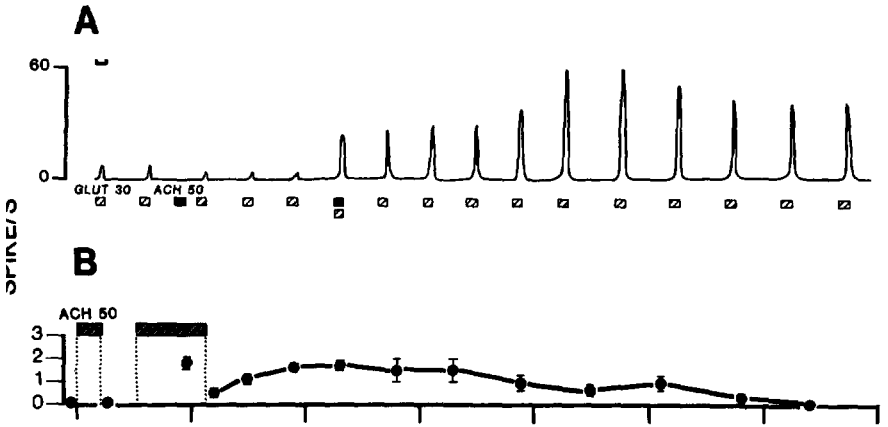

FIGURE 4. *Prolonged increases in excitability of somatosensory cortical neurons following iontophoretic application of ACh*

NOTE: (A) The experimental paradigm used to test the altered excitability to glutamate produced by ACh. During the Interval when repeated pulses of glutamate were released from one barrel, the ACh could be delivered from another barrel to see how it changed excitability. (B) The long-term enhancement of neuronal excitability when ACh was administered to a somatosensory cortical neuron during somatic stimulation. Before treatment, there was no response. When ACh was administered between stimuli, there was no enhancement. Subsequently, when ACh was administered during stimulation, the responses were enhanced severalfold and remained elevated for about 45 minutes. The calibration bar In part (A) indicates 1 minute.

Although ACh treatment produced rather dramatic effects in some cells, not all cells were affected by ACh; only about one-third of the cells underwent prolonged changes in excitabi1ity. For others, changes endured only as long as ACh was present, and for some there was neither a prolonged nor a short-term effect of ACh. Occasionally, ACh treatment reduced the excitability of somatosensory cortical neurons.

Thus, ACh may act as a permissive agent that can bring about selective enhancement of those neuronal responses that most strongly excite a cell. However, ACh itself does not cause the enhancement: the enhancement of a neuronal response depends upon excitatory input that is present while ACh is present, and only those inputs activated while ACh is present are enhanced. When this mechanism is combined with the release from inhfbition that follows deafferentation (allowing neurons to respond to previously ineffective inputs), it may play an important role in building new receptive fields for deafferented neurons. The release from inhibition that follows deafferentation could allow many additional inputs to drive the cell; then, those inputs that are actually

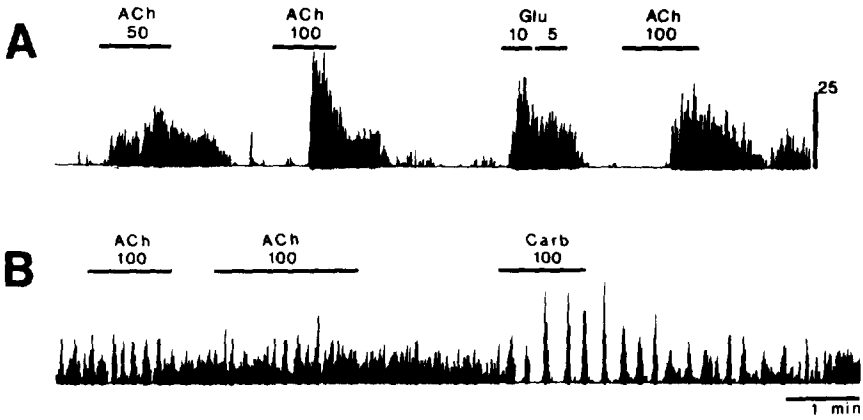

FIGURE 5. *Comparison of the effects of acetylcholine in normal and partially deafferented cortex*

NOTE: (A) A rate meter record of the excitatory responses to ACh and glutamate obtained from neurons In normal rat hindlimb somatosensory cortex. (B) A rate meter record of the excitatory effect of ACh and carbachol In deafferented rat hindlimb somatosensory cortex.

used would be the ones that are enhanced. This mechanism requires that ACh be released following deafferentation or that binding sites for ACh on the neurons in deafferented cortex become more numerous. Both possibilities remain to be explored.

CONCLUSION

The receptive field of a cortical neuron consists of a balance of excitation and inhibition which allows only a small fraction of the excitatory drive to be expressed at any one time. Often the inhibition appears to be mediated by GABA. In some cases, it is sufficiently powerful to completely prevent a cell from being driven from the periphery. The static organization of the receptive field, as seen in tests with bicuculline, suggests that the balance of excitation and inhibition sharpens a somatotopic order that is only roughly specified by the underlying pattern of partially shifted, overlapping thalamic arbours and axonal processes of cortical neurons. The resolution of the somatotopic map depends upon postsynaptic inhibitory processes.

The dynamic properties of receptive fields of cortical neurons may be attributed to transient changes in the local balance of excitation and inhibition that cause the excitatory focus of a neuron to shift. Similarly, the excitatory drive on a previously silent neuron may be enhanced by shifts in GABA-mediated inhibition until some part of the excitatory drive is expressed by the discharge of that cell. Obviously, the activity of inhibitory interneurons will play a key role in these rapid changes.

ACh can also play an important role in the dynamic processes of somatosensory cortex because it is released by brain stem mechanisms during motivated behaviors and states of selective attention. Although ACh may have a slower time course (measured in seconds rather than the milliseconds attributed to GABA-mediated processes), in its presence the excitability of most neurons is enhanced, and previously silent neurons become active, following a time course compatible with ACh involvement in cortical functions related to motivated behaviors.

ACh can also play a role in the even slower, longer term changes in excitability of somatosensory cortical neurons that occur following deafferentation. When neurons are depolarized in the presence of ACh, they become more responsive, for long periods after ACh is removed, to those inputs which produced the original excitation, thereby allowing ACh to bring about potentially important long-term changes in cortical organization.

It is clear from recent work on other neurotransmitters that substances such as noradrenalin, serotonin, and dopamine can also modulate neuronal excitability as does ACh. Thus, there are several routes by which drugs may alter the responsiveness of neurons on a long-term basis. These findings demonstrate that, in order to detect some of the plastic changes produced by drugs, it is often necessary to study neurons that are responding to their natural inputs. Moreover, if the system can be "stressed," drug effects which were not apparent in the normally functioning system may be uncovered.

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