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Opioids in the Hippocampus

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Opioids in the Hippocampus

Editors:

Jacqueline F. McGinty, Ph.D.
East Carolina University School of Medicine
Greenville, NC

David P. Friedman, Ph.D.
Division of Preclinical Research
National Institute on Drug Abuse

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Opioids in the Hippocampus

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Preface

The discovery in the middle 1970s of opiate receptors and endogenous opioid peptides was clearly one of the landmark events in the history of neuroscience. Studies designed to localize and characterize both the receptors and the peptides and to reveal their functional significance have grown at nearly an exponential rate since that time. Not surprisingly, a good deal of that effort has focused on the hippocampal formation. Its relatively simple, laminated, and well-described structure have made it a key model for studying many aspects of neural processing in the forebrain. Because different functional components exist almost exclusively in separate structural compartments, the hippocampus presents an excellent opportunity to correlate structure with function. As a result, it was not long after the initial discoveries of brain opioid systems that effects of oploids on hippocampal electrophysiology were reported.

The importance of the hippocampus far exceeds its use as a model, however, because the hippocampus acts as one important gateway by which sensory information that has been processed by the neocortex gains access to the limbic system. The hippocampus therefore plays a key role in memory, even showing the ability to control its own input and responses based on previous experience. In addition, the hippocampus appears to be a highly excitable structure with an unusual vulnerability to seizures. For a variety of reasons, then, the hippocampus has drawn the attention of neuroscientists.

Those who wished to understand the role of opioids and their receptors in neural processing have been among the group studying the hippocampus, and this monograph summarizes their first decade of work. As the reports in this volume show, this decade has indeed been a profitable one. The neural elements containing opioids and the particular forms of the peptides they contain are now

known. The distributions of particular subclasses of opioid receptors have been described on a regional and laminar basis, and the anatomical relationships of these receptors to the peptides that presumably bind to them are also known. At the cellular level, there is a good understanding of how opioids may control the excitability of some kinds of hippocampal neurons, and the receptors that mediate these effects have in some cases also been described. Tools have been developed that now allow us to examine within specific hippocampal pathways the metabolic control of both opioid peptide content and the genetic message leading to the synthesis of the propeptides from which hippocampal enkephalins and dynorphins are derived. In addition, the relations of opioid peptides to other transmitters and neurally active substances that may be contained in those pathways are also being described. Even some of the possible behavioral effects of opioids acting in the hippocampus have been carefully scrutinized.

This progress, while impressive, serves to highlight what we still need to learn. Most importantly, the physiological conditions that lead to the release of opioid peptides from axonal terminations and the effects elicited under those conditions remain to be revealed. These questions must be considered the central mysteries about opioids in the hippocampus. In addition, many more specific questions still need to be answered. For example, although we know the regional and laminar distribution of opioid peptides and their recepton, these do not always coincide. Thus, there are places with peptide but no receptors and vice versa. The meaning of this mismatch is still unclear. Moreover, we have not directly demonstrated the neural elements on which the receptors reside.

Most of the studies of cellular electrophysiology have concentrated on the pyramidal cells of the CA1 region of the hippocampus. We need to extend our understanding to other cell types and hippocampal regions. CA3 and the dentate gyrus are both coming under scrutiny, but further efforts are required. The dentate gyrus is especially crucial because it is the major recipient of the neocortical input that is relayed through the entorhinal cortex via the perforant path, and because it is the origin of one of the major opioid-containing pathways in the hippocampus, the mossy fiber system.

Our models of metabolic control are still new, and future needs require that they become even more sophisticated and sensitive so

that they may reveal the control mechanisms operating under physiological conditions. Finally, the role of the hippocampus in the myriad of opioid- and opiate-modulated behavioral effects requires much study, because there is still so much we do not know.

The first 10 years of study of opioids in the hippocampus have produced a solid base of knowledge and any number of interesting leads for the future. For the most part, however, the “simple” questions have been answered. Many of the gaps in our knowledge, therefore, represent opportunities for the study of difficult problems. The chapters that follow do an admirable job in explaining for us the current state of our knowledge and where future challenges lie.

David P. Friedman, Ph.D.
Division of Preclinical Research

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What We Know and Still Need To Learn About Opioids in the Hippocampus

Jacqueline F. McGinty

The study of opioid peptides and their receptors in the hippocampus integrates two major areas of fascination for neuroscientists. The current level of interest in hippocampal organization and functioning is demonstrated by the presentation of greater than 250 abstracts on the subject at the 1986 Society for Neuroscience meeting. This number is comparable to the number of abstracts at this meeting which focused on opiates and opioid peptides. A significant number of those abstracts represent the most current work of the participants in the "Opioids in the Hippocampus" workshop presented at that meeting. This volume summarizes those presentations, which include data on the anatomical, physiological, pharmacological, and behavioral aspects of the proenkephalin and prodynorphin systems in the hippocampal formation (HF, a term which includes the hippocampus proper and the dentate gyrus; see below). The authors have integrated the information accumulated on opioid peptides during the last dozen years with the body of knowledge accumulated on the neurobiology of the hippocampal formation during the last century. Together the data point to the possibility that opioid peptides are intimately involved in the molecular mechanisms underlying learning and memory and seizure susceptibility in the hippocampus.

In order to grasp the significance of the distribution of opioid peptides and receptors in the HF, it is necessary to have a basic appreciation of hippocampal structure and circuitry. The HF is a curved structure which must be sectioned in the frontal plane rostrally and in the horizontal plane caudally in order to retain the characteristic "laminar" relationship between the major cellular

layers throughout the structure. Figure 1 illustrates these relationships in a frontal section through the dorsal HF. The basic route of information flow through the HF, which is most relevant to the location of opioid-containing neurons, is via a trisynaptic excitatory chain. This chain originates in the entorhinal cortex, which projects highly processed, polymodal sensory information from the entire cerebral cortex through the perforant pathway to the granule cells in the dentate gyrus of the HF.

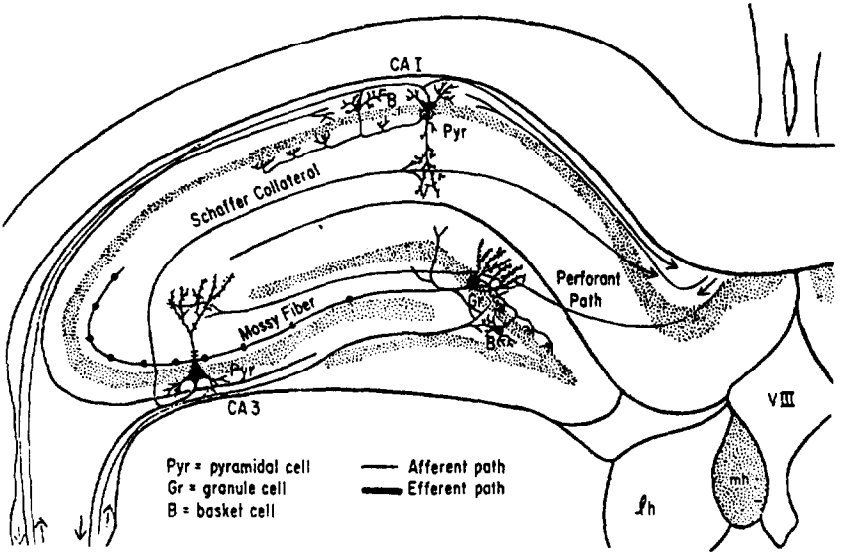

FIGURE 1. *A schematic illustration of a coronal section through the dorsal HF of the rat*

NOTE: The trisynaptlc excitatory chain consists of the perforant path arising in the entorhinal cortex, the granule cell-mossy fiber pathway innervating the CA4-3 cell fieid, and the Schaffer collateral pathway innervating the CA1 pyramidal cells.

SOURCE: Modified from Henriksen et al. 1982, Copyright 1982, The New York Academy of Sciences.

The granule cells are the second link in the chain: they project their axons, the mossy fibers, to the proximal apical dendrites of the pyramidal cells in the stratum lucidum of the CA3 (inferior)

region of the HF. The CA3 pyramids (the third link) not only project their axons out of the HF but also send a branch (the Schaffer collateral) to the pyramidal cell dendrites in the stratum radiatum of the CA1 (superior) region of the HF. In turn, the CA1 pyramids project axons out of the HF primarily through the fornix. Inhibition mediated by gamma-aminobutyric acid (GABA)- and/or peptide-containing interneurons (basket cells) whose axons surround the granule and pyramidal cells is a major control over HF excitability. Without this tonic inhibition, seizures would be free to march through the uninhibited hippocampus via the trisynaptic excitatory chain.

In studying the electrophysiological responsiveness of the HF, one must not assume that the entire structure is homogeneous from the dorsal through the ventral pole. Recently, it has become clear, for instance, that the perforant pathway from the entorhinal cortex into the HF is neurochemically as well as topographically heterogeneous, and that the threshold for excitability in the ventral pole is lower than that in the dorsal pole.

In the early 1980s, proenkephalin- and prodynorphin- (but not pro-opiomelanocortin-) containing neurons were found in the HF of rats by immunocytochemistry (Gall et al. 1981; Gall, this volume; McGinty et al. 1933; McGinty et al. 1994). Three different systems in the rat HF contain enkephalin immunoreactivity: two of the three major excitatory pathways, the perforant and mossy fiber systems (figure 2), and interneurons scattered throughout all layers of the HF (Gall et al. 1981). Subsequent to Gall's observations, a large concentration of prodynorphin peptides was discovered exclusively in the mossy fiber path which, under normal conditions, contains less proenkephalin than prodynorphin (Weber et al. 1982; McGinty et al. 1983; Gall, this volume: Herkenham and McLean, this volume). More recently, Gall (this volume) has observed some species differences in the enkephalin distribution in the HF, particularly a lack of enkephalin immunoreactivity in the perforant pathway in primates.

The realization that both proenkephalin and prodynorphin are contained in the mossy fiber path raised a number of interesting questions about opioid peptide interactions in the hippocampus. For example, are prodynorphin and proenkephalin both expressed in the same dentate granule cells? We explored this question with double antibody immunocytochemistry at the light microscopic level in

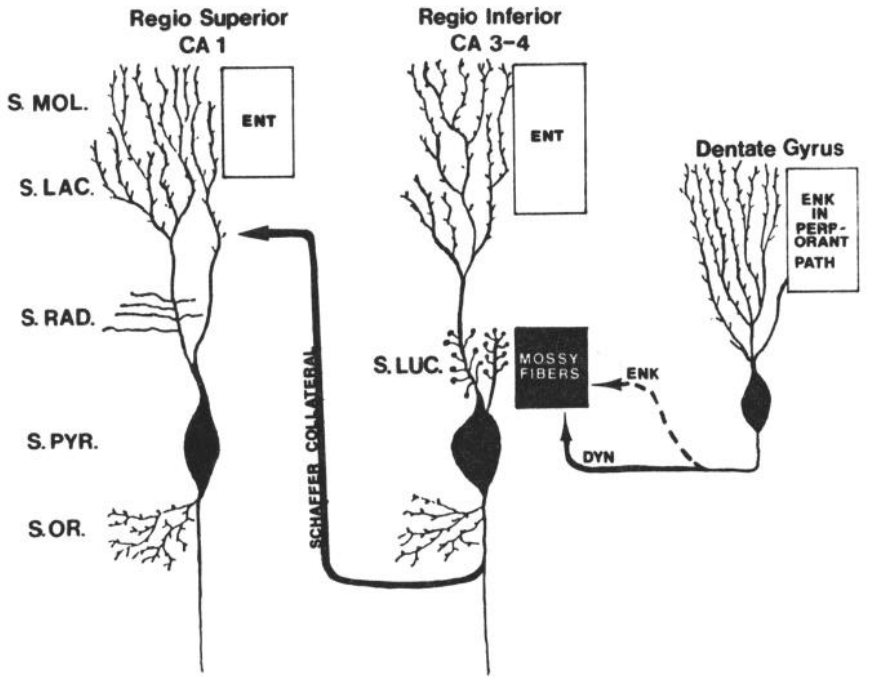

FIGURE 2. *A schematic illustratio depicting the three major neuronal types in the trisynaptic chain, the dentate granule cells, and the CA3 and CA1 pyramidal cells*

NOTE: Proenkephalin- containing fibers in the perforant pathyway innervate the proenkephalin- and prodynorphin-containing dentate granule cells. The granule cells givee rise to mossy fibers.

SOURCE: Modified from Altman et al. 1973, Copyright 1973, Academic Press.

colchicine-treated rats (McGinty 1985). Colchicine injected directly into the HF causes granule cell death, but when injected into the lateral ventricle, it causes axoplasmic transport blockade and can be used to reveal immunoreactive cell bodies throughout the central nervous system. In preparations where granule cells were visible, we were unable to find any granule cells which stained with antisera against products of both prodynorphin (dynorphin B) and proenkephalin (BAM22P). More recent *in situ* hybridization histochemical data have demonstrated that most if not all dentate

granule cells contain prodynorphin messenger RNA (mRNA) (Morris et al. 1988; Young, unpublished observations), but relatively few granule cells in a resting or nonstimulated state have been observed to contain proenkephalin mRNA (Young, unpublished observations). These results do not rule out the possibility that granule cells are capable of expressing both prodynorphin and proenkephalin peptides under certain physiological conditions. In fact, as pointed out by Gall (this volume) and Hong et al. (this volume), mossy fibers may be capable of synthesizing and releasing different combinations of neuroactive substances in response to changes in physiological activity.

Another question pertaining to the cellular localization of opioid peptides is whether or not enkephalins or dynorphins in granule cells and mossy fibers are colocalized with and released with other synaptically related molecules such as zinc, excitatory amino acids, or, in some species, cholecystokinin (Gall, this volume). Chavkin et al. (1983) have demonstrated that opioid peptides are released from hippocampal slices in a Ca^{+}-dependent manner by K^{+} depolarization or by the excitatory amino acid analogue kainic acid. Kainic acid also releases opioid peptides (Hong et al., this volume) and zinc (McGinty et al., in press) from mossy fibers *in vivo.* If other such substances are released along with opioids when mossy fibers are activated, investigations of their physiological effects should be pursued by testing their <u>interactions</u> on pyramidal and granule cell excitability in addition to examining the effects of each agent alone. Chavkin et al. (this volume) and Segal (this volume) introduce their most recent experiments in which the actions of enkephalins in the hippocampus are blocked with an excitatory amino acid antagonist (see below).

Two electrophysiological observations in rats initially attracted attention to the nature of opiate actions in the hippocampus (reviewed by Henriksen et al. (this volume) and Chavkin et al. (this volume)). First, intracerebroventricular (ICV) infusions of beta-endorphin or met^{5}-enkephalin into unanesthetized rats were found to induce electroencephalographic seizure activity in the hippocampus and amygdala in the absence of frank motor convulsions (Urca et al. 1977; Henriksen et al. 1978). These “limbic seizures” were accompanied by a frozen posture interrupted by “wet dog shakes,” a behavior which also occurs during opiate withdrawal in rodents. According to Hong et al. (this volume), the occurrence of wet dog shakes appears to depend on the actions of enkephalin on

or in the granule cell-mossy fiber pathway. Second, Nicoll and colleagues (1977) demonstrated a unique excitatory effect of electrophoresed enkephalin on individual hippocampal pyramidal cells. Only inhibitory actions had been recorded in other regions of the mammalian brain (Nicoll et al. 1977). Intracellular recordings (Zieglgänsberger et al. 1979), however, were unable to demonstrate the changes in pyramidal cell membrane polarization which should have occurred if opiate receptors on pyramidal cells were mediating the excitation. Zieglgänsberger and colleagues reconciled these two apparently discrepant findings by hypothesizing that inhibitory GABA-containing neurons intervened between inhibitory enkephalin-containing neurons and hippocampal pyramidal cells. In this scenario, when enkephalin is released, it would cause inhibition of GABA release and a resulting disinhibition of pyramidal cells. Recently it has been learned that enkephalin analogues do decrease the firing rates (Lee et al. 1980) of and hyperpolarize the membranes of CA1 intemeurons by increasing potassium conductance (Nicoll and Madison 1984). The study of the effects of opioid peptides in the dentate gyrus has a much shorter history; progress in this area is reported by Henriksen et al. (this volume) and Chavkin et al. (this volume).

To date, the enkephalin-GABA-pyramidal cell circuitry proposed by electrophysiologists in CA1 has neither been demonstrated nor refuted by morphological data. A demonstration of monosynaptic connections between enkephalin- and GABA-containing cells would require immunostaining for GABA (or glutamic acid decarboxylase, which synthesizes GABA from glutamate) and enkephalin simultaneously at the electron microscopic level. There is not yet, however, even a published account of enkephalin immunoreactivity alone in the hippocampus at the ultrastructural level. In other sytems (teleost and avian retina), it has been shown that exogenously applied enkephalins inhibit the K^+-evoked release of GABA from amacrine cells and that enkephalin is localized in some amacrine cells which take up [^{3}H]GABA (Chin and Lam 1982).

Until the localization of enkephalin and GABA at the ultrastructural level is performed together successfully, we will not know what class of hippocampal neurons have oploid receptors on their plasma membranes, and, as a result, we will not know by what mechanism enkephalin excites pyramidal cells. Although receptor autoradiography has provided us with detailed maps of the distributions of the mu, delta, and kappa opioid receptor subtypes within the

different fields and layers of the HF (Herkenham and McLean, this volume), the resolution of these receptor maps does not yet allow us to discern on what cell types the receptors are located. We still do not know if GABA-containing cells and/or pyramidal cells possess opioid receptors. What we do know is that the pyramidal cell layer, which is only sparsely innervated by opioidergic terminals in many species, contains the densest concentration of opioid receptor subtypes: whereas the mossy fiber layer, which contains the densest concentration of opioidergic terminals, has few, if any, opiate receptors. In other species, the distribution of opioid-peptide-containing terminals remains constant, but the distribution of opioid receptors is different. Herkenham and McLean (this volume) discuss the implications of this receptor-peptide "mismatching" in several species and provide some indication of which receptor subtypes may be in a position to mediate opioid peptide actions in different hippocampal regions.

The actions of hippocampal dynorphins on opioid receptor sub populations are also the subject of vigorous investigation. In contrast to the seizurogenic effects of met^5-enkephalin and beta-endorphin after ICV infusion, and their excitatory effects on single pyramidal cells, dynorphins not only do not produce seizures (Henriksen et al. 1982), but they also appear to have naloxons reversible excitatory as well as non-naloxone-reversible inhibitory actions at the single-cell level (Henriksen et al. 1982; Gruol et al. 1983; Moises and Walker 1985). Dynorphin effects, however, are mixed or are not very potent on pyramidal cells in the CA3 region of the hippocampus, which contains the most dynorphin-immunostained terminals. The paper by Chavkin et al. (this volume) explores the current status of this paradox by addressing what oploid receptor subtype(s) are activated by dynorphins in various regions of the hippocampus. Chavkin and colleagues replace the earlier hope that each class of opioid peptide would act via one class of opioid receptor with evidence that each peptide probably acts to different degrees on whatever receptor subtype(s) are present.

The presence of opioid peptides in excitatory pathways of the HF has led to explorations of the alterations in the metabolism of endogenous opioid peptides in response to hippocampal hyperexcitation. Hong et al. (1979) first demonstrated that kainic acid administration caused a seizure-linked depletion followed by a robust increase in met^5-enkephalin levels in the hippocampus. Since then, Hong's group, in collaboration with my own, has shown

that recovery of mossy fiber and perforant path proenkephalin and prodynorphin peptide and mRNA levels in response to seizures elicited by kainic acid, electroconvulsive shock, or kindling occur with very different time courses. These data complement the findings of Gall (this volume), who has shown that a lesion of the dentate hilus in mice also causes seizures that release opioids and cholecystokinin from the mossy fibers. In all of these seizure models, each peptide has a different rate of return to normal levels in the postictal period. Taken together, these studies indicate differential regulation of opioid peptide gene expression in hippocampal pathways and differential control of release of opioid peptides from the axons comprising these pathways. They have provided us with models in which to study the physiological consequences of short- and long-term alterations in peptide content and metabolism in the HF and hold promise for uncovering the role(s) of opioid peptides in hippocampal excitability.

Finally, in what way can the actions of hippocampal opioid peptides be associated with the role of the hippocampus in overall brain function? Chavkin et al. (this volume) and Segal (this volume) report their most recent data on the mediation of a specific component of opioid excitation of pyramidal cells in CA1 by receptors for the excitatory amino acid N-methyl-D-aspartate (NMDA). Activation of NMDA receptors occurs in long-term potentiation, the putative substrate for hippocampal learning and memory processing. Indeed, Segal (this volume) describes exciting new data showing that mu opioid receptor activation enables the expression of long-term excitatory postsynapatic potentials evoked by associating afferent stimulation with depolarization of CA1 pyramidal cells, an illustration of a so-called Hebbian synapse. Thus, by enhancing the activity of excitatory neurotransmitters, possibly by decreasing GABA-mediated inhibitory tone at specific synapses, opioid peptides may be essential to the very fabric of hippocampal synaptic plasticity in which memory is stored through changes in the efficacy of transmission among elements.

Based solely on the fact that opioid peptides are generously rep resented in important hippocampal circuits, one would anticipate that they would play a significant role in hippocampal-associated behaviors. Gallagher (this volume) reviews the evidence that exogenous opioid agonists diminish, and opioid antagonists improve, behavioral performance on memory tasks known to be dependent on integrity of the HF. However, it is not clear from these data that

opioid peptidergic circuits in the hippocampus are involved in these behaviors or that the tasks described lead to the release of endogenous opioids. A stronger indication that endogenous hlppocampal opioids are involved in spatial learning is provided by the data of Collier and Routtenberg (1984), showing that naloxone can block the forgetting of a learned spatial task, the disruption of which was induced by Stimulation of the dentate gyrus, presumably leading to the release of opioid peptides. Gallagher also presents a line of reasoning based on data from her laboratory indicating that cholinergic afferents from the septal nuclei to the hippocampus, which are important for the acquisition and retention of spatial tasks, are under opioid control. Finally, preliminary data from Gallagher's laboratory are presented which positively correlate changes in hippocampal opioid peptide levels with the acquisition and performance of a spatial task in a radial arm maze.

The authors contributing to this monograph hope that they were successful in synthesizing in a thoughtful and useful way the most current multidisciplinary data on the cellular localization and actions of opioid peptides in the HF. In addition, we hope we have achieved our goal of highlighting the most exciting and fruitful areas for future research, which will address not only the role of opioid peptides in the hippocampus but in the overall scheme of brain functioning as well.

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AUTHOR

Jacqueline F. McGinty, Ph.D.
Department of Anatomy and
Cell Biology
East Carolina University
School of Medicine
Greenville, NC 27858

Localization and Seizure-Induced Alterations of Opioid Peptides and CCK in the Hippocampus

Christine M. Gall

INTRODUCTION

As a consequence of its very simple laminated anatomy, the hippocampal formation has come to occupy a central position in neuroscience research as a model system for the analysis of cellular operations in the forebrain. As discussed in the various contributions to this volume, this has included use of the hippocampus as a vehicle for analyses of the role of opioid peptides in neuronal processing and behavior. Evaluation of the function of specific neuropeptides in the hippocampus, as elsewhere, must begin with consideration of such fundamental issues as their distribution, the extent to which this distribution is consistent across species, and the ontogeny of the peptides in the context of the morphological and physiological development of the structure. Optimally, one would also like to identify physiological processes which influence, and/or are Influenced by, the peptides under investigation. In the following pages, I will discuss the above points with regard to enkephalin, dynorphln, and cholecystokinin (CCK) in the hippocampus.

LOCALIZATION Of OPIOID PEPTIDES IN THE HIPPOCAMPUS

As described in my original report (Gail et al. 1981 b) and verified by subsequent immunocytochemical and biochemical studies, enkephalin immunoreactivity is localized within three distinct axonal systems in the rat hippocampal formation: the mossy fiber axons of the dentate gyrus granule cells which innervate pyramidal cells of region CA3; a thin band of axons distributed along the border of the stratum radiatum and the stratum lacunosum-moleculare of region CA1; and perforant path axons which arise from the lateral

entorhinal/perirhinal cortices, distribute within the Stratum iacunosum-moleculare, and innervate the distal third of the dentate gyrus molecular layer. The latter system is most dense in the temporal hippocampus. Enkephalin immunoreactivity is also observed within sparsely distributed perikarya throughout the hippocampus proper and (in untreated rats) within only a small number of morphologically characteristic dentate gyrus granule ceils. Dynorphin exhibits a much more restricted distribution in the rat hippocampus, being localized within the mossy fiber axonal system (McGinty et al. 1983) and very few scattered, multipolar neurons. Initially, the reported codistribution of [leu^5]enkephalin and dynorphin immunoreactivities within the mossy fiber system aroused concern that some of the enkephalin immunostaining may have been attributable to cross-reactivity of the enkephalin antisera with dynorphin (McGinty et al. 1993). However, subsequent studies have demonstrated that the dentate gyrus granule cells synthesize, and the mossy fiber axons contain, methionine enkephalin as well as the proenkephalin A fragments methionine-enkephalin-ArgPhe, methionine-enkephaiin-ArgGlyLeu, BAM 18, and BAM 22 (McGinty et al. 1984; White et al. 1987).

The localization of enkephalin and dynorphin within the mossy fiber system is invariably conserved across all species thus far examined, including rat, mouse, hamster, vole, European hedgehog, gerbil, guinea pig, cat, tree shrew, and Old World monkey (Gall et al. 1981b; Fitzpatrick and Johnson 1981; Tielen et al. 1982; Stengaard-Pedersen et al. 1983; Gall and Lauterborn, unpublished observations). In contrast, subtle interspecies differences are evident in the distribution of enkephalin within strata lacunosum and moleculare of the hippocampus proper, and these seem associated with the more striking differences in the localization of enkephalin within the perforant path afferents to the dentate gyrus. For example, in stratum lacunosum-moleculare of the rat ventral hippocampus, terminal-like immunoreactive puncta are most densely distributed within a zone extending from the subiculum into the adjacent region of CA1, and within region CA3 in a field continuous with the dentate gyrus outer molecular layer (OML) (figures 1 and 2). In contrast, there is very little immunostaining in the hamster stratum lacunosum-moleculare of CA3, but there is a rather dense patch of immunoreactive puncta within this lamina at the CA2 limit of region CA1. In the hamster, there is also very little immunoreactivity within the dentate gyrus OML in the guinea pig, enkephalin immunoreactivity is only prominent in the extreme

temporal tip of the CA3 stratum moleculare and in the dentate gyrus OML (Tielen et al. 1982), while in the hedgehog (Stengaard-Pedersen et al. 1983) and the tree shrew (Fitzpatrick and Johnson 1981), enkephalin immunoreactivity has not been observed in either of these laminae.

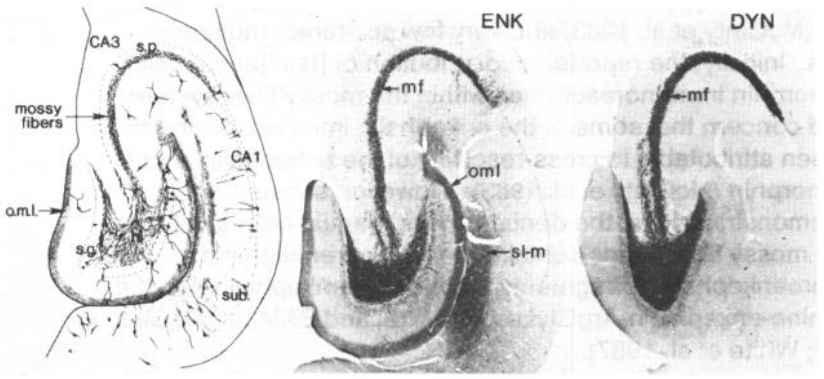

FIGURE 1. *Illustrations of the distribution of enkephalin-like and dynorphin-like immunoreactivity*

NOTE: Enkephalln (ENK)-like (drawing and left micrograph) and dynorphin (DYN)-like immunoreactivity (tight micrograph) in a horizontal section through the ventral rat hippocampus (peroxidase antiperoxidase technique). Abbreviations: sl-m, stratum lacunosum-moleculare; sg, stratum granulosum: mf, mossy fibers; oml, outer molecular layer: sp, stratum pyramidale.

In the Old World monkey *Macaca fascicularis,* enkephalin-immunoreactive mossy fibers are present within the stratum lucidum, but there is no clear lamination of enkephalin-positive elements in the molecular layers of the hippocampus proper. Only a few scattered immunoreactive axons and terminal puncta are seen within the stratum radiatum and, with somewhat greater density, within the stratum moleculare. Similarly, in the dentate gyrus molecular layer, enkephalin is localized within a few broadly distributed varicose axons: no prominent or laminated immunoreactive afferent systems are seen (figure 2).

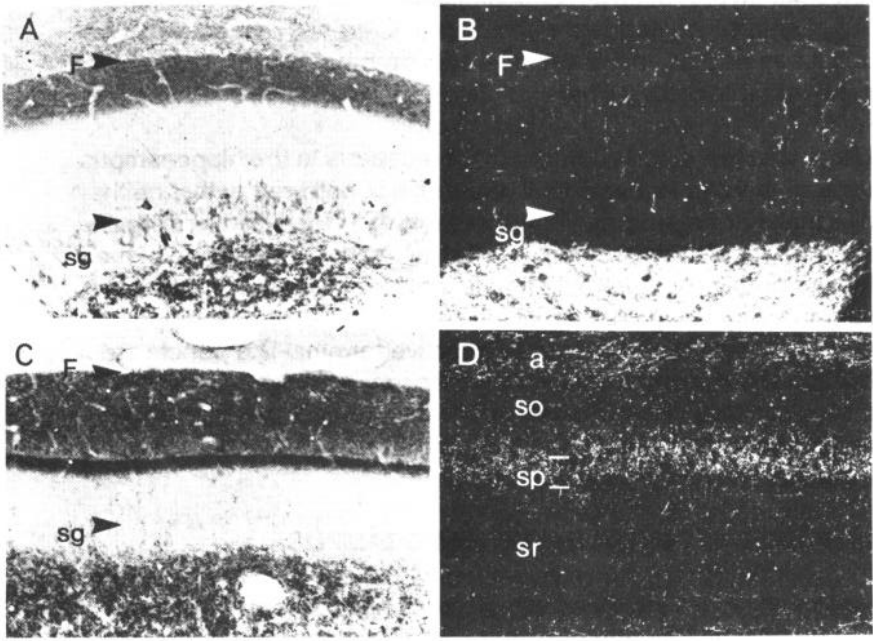

FIGURE 2. *Differences in the distribution of enkephalin immunoreactivity in the ventral dentate gyrus of the rat, the monkey, and the cat*

NOTE: Light-field (A and C) and dark-field (D and E) photomicrogmphs illustrating differences in the distribution of enkephalin immunoreactivity in the ventral dentate gyrus of the rat (A), the monkey (B), and the cat (C), and the localization of fine punctate immunoreactivity overlying the stratum pyramidale (sp) of the cat hippocampal region CA1. In A, B, and C, the arrowheads bracket the dentate gyrus molecular layer which can be seen to contain one distal outer molecular layer band of immunoreactivity in the rat (A), two bands of immunoreactivity in the cat (C), and no Laminated immunoreactive systems in the monkey. The mossy A ber system, in the hilus below the stratum granulosum (sg), is clearly immunoreactive in all three animals (A, B. and C). Abbreviations: a. alveus; F. hippocampal fissure: so, stratum oriens: sr, stratum radiatum.

In contrast to all other animals studied, the cat has two distinct and dense bands of enkephalin immunoreactivity occupying the middle and outer thirds of the dentate gyrus molecular layer across its

full septotemporal extent (figure 2). The molecular layer is considered to be composed of three laminae defined by the laminated termination of afferent systems: the inner, middle, and outer molecular layers receive hippocampal commissural/associational, medial entorhinal, and lateral entorhinal afferents, respectively. As such it seems that, in the cat, afferents from both the medial and lateral entorhinal cortex have relatively high enkephalin content.

In regard to the distibution of opioid receptors in the hippocampus (Herkenham and McLean, this volume), it is noteworthy that neither enkephalin nor dynorphin immunoreactivtty is localized to a significant number of axons or cells within the stratum pyramidale of the hippocampus proper in most animals. The only known exception to this generality is, again, the cat. In region CA1 of the cat hippocampus, fine enkephalin-immunoreactive terminal-like puncta are distributed quite precisely over the stratum pyramidale and the adjacent portion of the stratum oriens in a manner reminiscent of the distribution of opioid ligand binding observed in other species (figure 2).

DISTRIBUTION OF CCK IN THE HIPPOCAMPUS

Significantly more interspecies variability has been noted in the hippocampal distribution of a third, nonopioid peptide, cholecystokinin octapeptide (CCK). CCK is of interest when considering hippocampal opioids in that this peptide is codistrlbuted with enkephalin and dynorphin in the mossy fiber system of some species, is reportedly antagonistic to opioid-mediated processes in other systems (Fads et al. 1983), and is influenced by the same physiological events which after hippocampal opioid synthesis *(vide infra).* CCK is reliably localized within scattered perikarya throughout the hippocampus proper and the dentate gyrus hilus, and within coarse pericellular axons in the stratum pyramidale. There are species differences in the density and distribution of CCK in the commissural/associational afferents to the dentate gyrus inner molecular layer, and the medial perforant path afferents to the dentate gyrus middle molecular layer (Stengaard-Pedersen et al. 1983; Gall 1984a; Gall et al. 1988). More pertinent here, CCK immunoreactivity has been localized within dentate gyrus granule cells and the axons of the mossy fiber system of guinea pig, European hedgehog (Stengaard-Pedersen et al. 1983; Gall 1984a), mouse (Gall et al. 1986), and *M. fascicularis* (Gall and Lauterborn, unpublished), but is

absent from this system in rat (Greenwood et al. 1981), gerbil, rabbit, and cat (Gall, unpublished).

The extent to which the three mossy fiber peptides are colocalized within individual mossy fiber terminal boutons, as opposed to being localized within separate elements within this system, is not as yet known. In a double-labelling study, McGinty (1985) found immunoreactivity for dynorphin B and the proenkephalin A product BAM 22 in separate granule cells in colchicine-treated rats. However, enkephalin synthesis and concentration In the dentate gyrus granule cells has been demonstrated to be exquisitely sensitive to recent physiological activity. In the normal state, without physiological stimulation of synthesis, only a fraction of the granule cells which are able to synthesize enkephalin contain detectable immunoreactivity (see below). Consequently, the absence of dynorphin/enkephalin colocalizatlon In the normal rat, or enkephalin/CCK colocalization in the normal mouse, does not necessarily mean that these peptides arise from biochemically distinct perikarya. It is possible that individual cells do not synthesize these peptides in detectable quantities all the time or at the same time. There is, in fact, evidence that the synthesis of mossy fiber peptides is independently, or possibly even reciprocally, regulated in ontogeny and adulthood, as will be discussed next.

ONTOGENY OF ENKEPHALIN, DYNORPHIN, AND CCK IMMUNOREACTIVITY IN THE HIPPOCAMPUS

Data on the ontogeny of opioid peptide immunoreactivity in the hippocampus, and most particularly within the hippocampal mossy fiber system, is directly relevant to the possible functions of the opioids in hippocampal physiology. In young rats, enkephalin immunoreactivity within the lateral perforant path afferents to the ventral dentate outer molecular layer is evident at the time of afferent ingrowth—4 days postnatal (dpn). In contrast, enkephalin is not detectable within the mossy fibers until about 12 dpn and, in both the rat and the mouse, is first seen in a few large, well-elaborated mossy fiber boutons (Gall 1984b; Gall et al. 1984). The mossy fibers are known to grow into the stratum lucidum of region CA3 by 3 dpn (Zimmer and Haug 1978), to have formed morphologically recognizable and functionally competent synapses by 5 dpn (Bliss et al. 1974; Sterling and Bliss 1978), and, only after synaptic contacts have been established, to slowly elaborate the large terminal boutons characteristic of the adult (Amaral and Dent 1981).

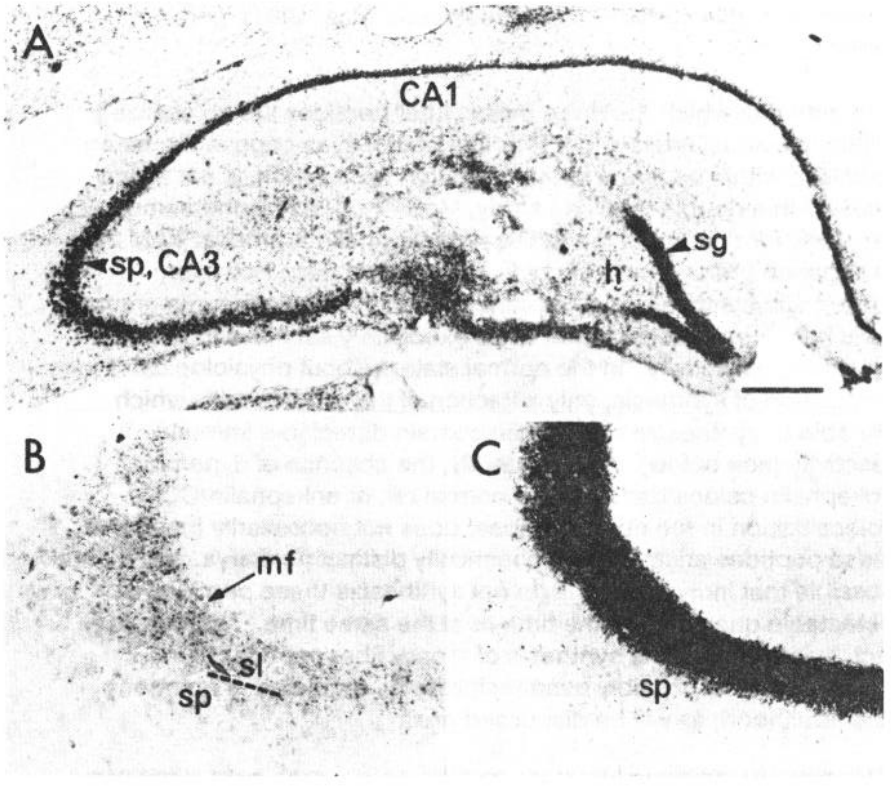

FIGURE 3. *The hilus lesion and seizure-induced changes in mossy fiber enkephalin immunoreactivity*

NOTE: Photomicrographs illustrating (A) the extent of the electrolytic hilus lesion found to initiate recurrent limbic seizuree activity (Nissl stain, coronal section from rat), and (Band C) the increase in enkaphalin lnmunostaining in the mossy fiber system (region CA3b) 4 days after a contralateral hilus lesion (C), relative to that seen in a paired, untreated rat (B). Abbreviations: h, hilus; mf, mossy fibers: sg, stratum granulosum: sl, stratum lucidum: sp, stratum pyramidale.

Considered in this context, it is clear that enkephalin is first detectable within the mossy fibers fairly late in the morphological development of the system and well after the establishment of synaptic transmission.

Dynorphin and, in the mouse, CCK both appear earlier in the development of the mossy fiber system, and closer to the apparent

onset of synaptic function. In the rat and the mouse, both peptides are first detectable on 6 dpn in small, indistinct puncta within the dentate gyrus hilus and the stratum lucidum (Gall 1984b). The immunoreactive elements within the stratum lucidum enlarge with age, and it is only at later time points (12 to 14 dpn) that both immunoreactivities are clearly localized within the large, irregularly shaped terminal boutons characteristic of the mossy fibers in the adult. Thus, in contrast to the development of enkephalin within this system, both dynorphin and CCK emerge very soon in the wake of synaptogenesis, suggesting that these peptides, but not enkephalin, may be present and active from the onset of synaptic transmission at the mossy fiber synapse.

EVIDENCE FOR THE REGULATION OF HIPPOCAMPAL NEUROPEPTIDE LEVELS BY PHYSIOLOGICAL ACTIVITY

Evidence that opioid peptide levels in the hippocampus and elsewhere are regulated to a significant extent by physiological activity is shown in studies of the effects of experimentally induced seizures. Hong and coworkers first demonstrated that, following recurrent seizures induced by either repeated electroconvulsive shock (Hong et al. 1979) or intracerebroventricular (ICV) injection of kainic acid (Hong et al. 1980), the level of enkephalin immunoreactivity in the hippocampus increases threefold as measured by radioimmunoassay (RIA) (Hong, this volume). These data provide an interpretation for the observation I had made that a small electrolytic lesion placed in the dentate gyrus hilus induced a large bilateral increase in mossy fiber enkephalin immunoreactivity (Gall et al. 1981 b). Since that time, a number of other seizure-producing treatments have been demonstrated to alter both enkephalin and dynorphin levels in the hippocampus. Work in my laboratory has focused on the effect of limbic seizures induced by either focal lesion placement in the dentate gyrus hilus or, for the purpose of comparison, ICV injection of kainic acid, on enkephalin, dynorphin, and CCK systems in rat and mouse hippocampi. Both treatments induce dramatic increases in enkephalin immunoreactivity in the hippocampus which are most prominently localized within the hippocampal mossy fiber system (Gall et al. 1981a). Although enkephalin immunoreactivity is clearly altered in other brain areas following these treatments, our work has centered on peptide changes in the mossy fibers due to the codistribution of three distinct peptide families within this one axonal system; the relatively high sensitivity of mossy fiber peptide levels to seizure activity;

and the anatomical suitability of this compact, well-characterized, and strictly unilateral axonal system to both biochemical and immunocytochemical analysis.

In the rat, placement of a small unilateral electrolytic lesion in the hilus of the dentate gyrus (figure 3) induces synchronous, bilateral epiieptiform activity in the hippocampus, which is first observed 1 to 2 hours postlesion and continues, with periodic full paroxysmal discharges, for approximately 10 hours thereafter. Throughout this period, the animals exhibit intermittent convulsive behavioral seizures reminiscent of those described to accompany kindled limbic seizures by Racine (1972). As such, three to seven episodes of stage 4 or 5 convulsive seizure (rearing with facial and forelimb clonus, rearing and falling), and more numerous displays of associated subconvulsive seizure behavior (forelimb clonus and wet dog shakes) are typically observed. This lesion directly severs a narrow rostrocaudal span of the strictly ipsilateral, and highly topographic, mossy fiber system within the lesioned hippocampus; the majority of the mossy fibers within the ipsilateral hippocampus and all of the mossy fibers within the contralateral hippocampus remain fully intact. Kaink acid induces more continuous seizure activity lasting 3 to 6 hours after an ICV injection, does not induce mossy fiber degeneration, but does result in the death of neurons within region CA3 of the rostral, ipsilateral hippocampus.

Enkephalin levels undergo a biphasic fluctuation following seizures induced by either treatment (Pico et al. 1985; Gall et al., in press: Kanamatsu et al. 1986b). In rats sacrificed 6 to 12 hours following a hilus lesion, enkephalin immunostaining is completely depleted from the mossy fiber system bilaterally. By 18 to 20 hours, immunoreactivity has returned to near-normal levels, and by 24 hours, immunostaining is clearly greater than that observed in paired controls. Enkephalin immunoreactivity in the mossy fibers continues to increase until it reaches an apparent maximum at 3 to 5 days postlesion, and thereafter decreases to normal by about postlesion day 10. At its maximal elevation, hippocampal enkephalin content reaches over fourfold above normal as measured by RIA. The time course for the fluctuations in hippocampal enkephalin content induced by the hilus lesion is comparable to that observed following ICV or intrastriatal injection of kalnic acid in our immunocytochemical material and in the reports of Hong et al. (1980) and Kanamatsu et al. (1988b). We find enkephalin levels are also transiently elevated in other areas following either the hilus

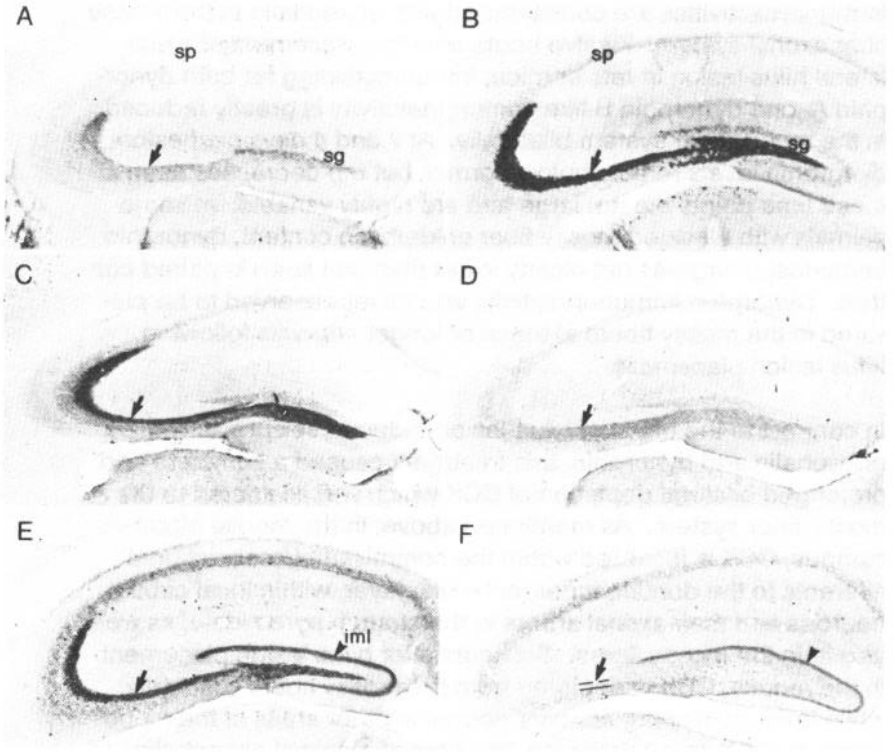

FIGURE 4. *Seizure-induced changes in mossy fiber enkephalin, dynorphin, and CCK immunoreactivities in mouse hippocampus*

NOTE: Photomicrographs of coronal sections through the mouse hippocampus illustrating the distribution and density of peroxidase antiperoxidase immunostaining for enkephalin (A and B), dynorphin (C and D), and CCK (E and F) in untreated control mice (A, C, and E) and paired mice sacrificed 4 days after the contralateral placement of a seizure-producing hilus lesion (B, D, and E). Abbreviations: iml, inner molecular layer; sg, stratum granulosum; sp, stratum pyramidale. Arrows indicate mossy fiber system.

lesion or kainic acid, including the temporoammonic/lateral perforant path, entorhinal cortex, lateral septum, and amygdala.

The effect of hilus-lesion-induced seizure activity on the regulation of mossy fiber enkephalin content is somewhat selective in that neither dynorphin nor CCK immunoreactivities are increased by this treatment. As mentioned above, dynorphin and, in the mouse, CCK

immunoreactivities are codistributed with enkephalin in the mossy fiber axonal system. Twelve hours after the placement of a unilateral hilus lesion in rats or mice, immunostaining for both dynorphin A- and dynorphin B-like immunoreactivity is greatly reduced in the mossy fiber system bilaterally. At 2 and 4 days postlesion, dynorphin levels remain below normal, but the decreases seen at these time points are not large and are highly variable: in some animals with elevated mossy fiber enkephalin content, dynorphin immunostaining was not clearly lower than that seen in paired controls. Dynorphin immunoreactivity was never observed to be elevated in the mossy fibers at these or longer intervals following hilus lesion placement.

In contrast to the effect of hilus-lesion-induced seizure activity on enkephalin and dynorphin, this treatment caused a complete and prolonged bilateral depletion of CCK which was restricted to the mossy fiber system. As mentioned above, in the mouse hippocampus, CCK is localized within the commissural/associational afferents to the dentate inner molecular layer, within local circuit neurons and their axonal arbors in the stratum pyramidale, as well as within the mossy fibers. Six hours after hilus lesion placement in the mouse, CCK is depleted from the mossy fibers bilaterally, while immunostaining appears normal in other areas of the hippocampus. At 4 days postlesion (the time of maximal enkephalin elevation), the pattern of CCK immunoreactivity remains unchanged: no immunoreactivity is apparent within the mossy fiber system, while other CCK systems stain normally. Low doses of kainic acid (0.05 to 0.1 µg, ICV) induce precisely the same alterations in mouse mossy fiber peptide immunoreactivity as those seen following the hilus lesion: an initial depletion of immunostaining for all three peptides; a return and supranormal elevation of enkephalin immunoreactivity which appears maximal 3 to 5 days posttreatment; a persistent depletion of CCK restricted to the mossy fibers; and a partial reduction in immunostaining for dynorphin (Gall 1984c).

Data on the duration of altered peptide immunoreactivity in the mouse following either the hilus lesion or kainic acid treatment serve to further dissociate the effects of seizures on mossy fiber enkephalin and CCK, and to indicate that aberrant peptide levels induced by seizures can become permanent. Unlike the rat, the duration of elevated mossy fiber enkephalin in the mouse is highly variable: while enkephalin immunostaining has returned to normal in most animals sacrificed 2 weeks posttreatment, in four mice

which initially exhibited particularly severe seizure behavior, enkephalin immunostaining was still dramatically elevated in the mossy fibers after 2 to 6 months' survival. The absence of CCK from the mossy fibers generally outlasted the enkephalin elevation. In a number of the experimental mice with normal enkephalin immunostaining 14 to 20 days posttreatment, CCK immunoreactivii had not yet returned to the mossy fiber system. Among the mice allowed 2 to 6 months' survival, all mice with normal enkephalin immunoreactivity also exhibited normal CCK immunoreactivity in the mossy fibers. Those few animals with a persistent elevation in mossy fiber enkephalin exhibited absolutely no immunostaining for CCK in this system (Gall et al. 1986).

Because enkephalin, dynorphin, and CCK are similarly depleted from the mossy fiber system during the recurrent seizure episode, one might argue that the dentate gyrus granule cells, which synthesize and release these peptides, are similarly stimulated by the epileptiform event. However, the postictal levels of the three mossy fiber peptides are altered in distinctly different fashions. Either the physiological activity or peptide depletion clearly stimulates a large increase in enkephalin content. The same processes lead to an equally dramatic, and somewhat more persistent, loss of mossy fiber CCK. Dynorphin levels are less severely affected, seeming only partially depressed in most experimental animals.

ALTERATIONS IN ENKEPHALIN SYNTHESIS FOLLOWING SEIZURE

There is now substantial evidence that the increase in enkephalin content observed in the hippocampus and elsewhere (Yoshikawa et al. 1985) following recurrent seizures is due to increased synthesis. Work done in my laboratory in collaboration with Drs. Jeff White and Jeff McKelvy (of State University of New York at Stony Brook) has specifically examined the effect of recurrent seizure activity induced by unilateral hilus lesion on enkephalin metabolism in the dentate gyrus granule cells which give rise to the mossy fiber axonal system. As reviewed earlier, following hilus lesion placement in the rat, enkephalin immunoreactivity increased within the mossy fiber axons bilaterally from 12 hours to 3 days postlesion. In order to determine the effect of this treatment on enkephalin synthesis within the hippocampus contralateral to the lesion, we injected ^{35}S-methionine by osmotic minipump into the dentate gyrus during the interval from 24 to 28 hours postlesion. The incorporation of the isotope into chromatographically identified

methionine-enkephalin, methionine-enkephalin-ArgPhe, methionine-enkephalin-ArgGlyLeu, and BAM 18 purified from region CA3 was measured at 30 hours postlesion. We found that there was a 14-fold greater incorporation of the radiolabel into methionine-enkephalin in the mossy fiber system of hilus-lesioned rats relative to paired controls, while there was no change in the ratio of the four proenkephalin A peptides (White et al. 1987). This indicates that hilus-lesion-induced seizure activity causes a large increase in proenkephalin A synthesis without a fundamental change in post-translational processing.

In a separate series of rats, we conducted Northern blot analysis to determine the effect of hilus-lesion-induced seizure activity on preproenkephalin messenger RNA (mRNA) content in the dentate gyrus contralateral to lesion placement (figure 5). We detected an increase in the single species of RNA hybridizing to our ^{32}P-labelled RNA probe as early as 3 hours posttesion, less than 2 hours after the onset of seizure activity. Preproenkephalin mRNA levels reached a maximal increase of about 24-fold above normal at 30 hours postlesion. By 4 days, message levels were only slightly higher than those observed in untreated rats, and by 10 days the experimental and control values were equivalent (White et al. 1987; White and Gall 1987). As expected if these increases in enkephalin message were induced by seizure activity rather than some other aspect of treatment, we found that maintenance of animals under anesthesia with the anticonvulsants sodium pentobarbital and phenytoin from lesion until sacrifice did, in fact, significantly block the hilus lesion induction of increased preproenkephalin mRNA in the dentate gyrus (figure 5).

The observation that recurrent seizure activity induces increased enkephalin immunoreactivity and synthesis is consistent with the results of other laboratories using different experimental manipulations including kainic acid injection (Kanamatsu et al. 1986b), repeated electroconvulsive shock (ECS) (Kanamatsu et al. 1986a), and chemical (pentylenetetrazol)- and amygdala-stimulation-induced kindling (Vindrola et al. 1984; ladarola et al. 1986). Together, these treatments have elicited enkephalin increases in the hippocampus, entorhinal cortex, amygdala, ventral pallidum, septum, hypothalamus, and substantia nigra, although there are differences in the reported distribution and magnitude of increased enkephalin immunoreactivity with different modes of seizure induction. Following either the hilus lesion or kainic acid, the greatest increase

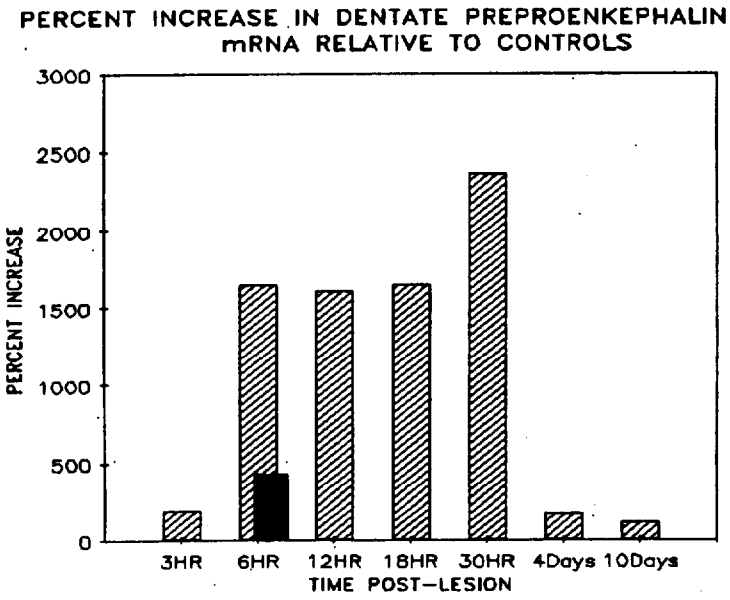

FIGURE 5. *Bar graph illustrating the time course of increased preproenkephalin A mRNA in the rat dentate gyrus contralateral to a hilus lesion*

NOTE: Each bar represents the mean densitometric value from autoradiograms of Northern blots for at least three animals at each time point. Cross-hatched bars indicate rats anesthetized during surgery with ketamlne/xylazlne; black bar indicates rats maintained under pentobarbital/phenytoin anesthesia from lesion until sacrifice.

in enkephalin immunoreactivity is localized in the mossy fiber system. In contrast, following ECS, elevated hippocampal enkephalin levels are greatest in the perforant path afferents from the entorhinal cortex. ECS induces the largest increases in enkephalin content in the hypothalamus, whereas enkephalin levels are unaffected in this area following kainic acid. It is noteworthy that all effective paradigms include recurrent seizure activity: Kanamatsu et al. (1986a) did not observe significantly increased hippocampal enkephalin content following less than six consecutive ECS treatments. In both the hilus lesion (White and Gall 1987) and kainic acid (Kanamatsu et al. 1986b) paradigms, increased synthesis, as indicated by increased preproenkephalin mRNA levels, begins

early in the period of recurrent seizure activity and, in the hilus lesion model, continues to increase for several hours after the termination of behavioral seizures.

The effect of seizures on both dynorphin and CCK is significantly less consistent across paradigms. While repeated ECS leads to increased dynorphin immunoreactivity in the substantia nigra, medial basal hypothalamus, and ventral pallidum (Kanamatsu 1986a), ECS, the hilus lesion, low doses of ICV kainic acid (present data), and stage 5 amygdaloid kindling (ladarola et al. 1986) effect a reduction in dynorphin in the mossy fibers lasting several days at least. High doses of kainic acid injected into the striatum reportedly induce a rapidly transient increase in mossy fiber dynorphin (Kanamatsu et al. 1986b). While both amygdaloid kindling (ladarola et al. 1986) and kainic acid (Meyer et al. 1986) have been reported to increase CCK immunoreactivity in the substantia nigra and hippocampus of rats, we have found that both the hilus lesion and kainic acid eliminate CCK from the mouse mossy fiber system. These results are not directly contradictory, however, in that CCK immunoreactivity is not localized within the mossy fibers of the rat.

CONCLUDING COMMENTS

It is now clear that two separate opioid peptide families, represented by enkephalin and dynorphin, are localized and, to a large extent, codistributed within the hippocampal formation. While both peptides are consistently observed within the dentate gyrus granule cells and their mossy fiber axons in a number of different mammals, there are substantial interspecies differences in the localization of enkephalin within the entorhinal afferents to the dentate gyrus and hippocampus proper.

Data on the ontogeny of and the effect of seizures on enkephalin, dynorphin, and CCK in the mossy fiber system suggest that these peptides are both independently regulated and differentially regulated by physiological activity, even though all three synthetic systems may coexist within individual neurons. In the untreated mouse, far more mossy fiber boutons contain dynorphin and CCK immunoreactivity than enkephalin immunoreactivity. Following recurrent seizures, the reverse is true: a high density of boutons contain enkephalin, while fewer dynorphin- and no CCK-immunoreactive boutons are found. The seizures seem to push the mossy

fiber system from “State-A” (enkephalin poor) to “State-B” (enkephalin rich). Put in these terms, these findings encourage speculation that individual granule cells might also have a State-A in which they actively synthesize dynorphin and, in some animals, CCK; and a State-B in which they actively synthesize enkephalin and not the other peptides. As suggested earlier, this would account for the localization of dynorphin B and the proenkephalin A product BAM 22 within separate granule cells (McGinty 1985), without leading to the conclusion that there are distinct populations of granule cells as characterized by their invariant synthesis of either enkephalin or dynorphin.

Taking this hypothesis one step further, the presence of dense enkephalin immunoreactivity in a subset of mossy fiber boutons in the untreated animal may reflect the differences in the recent physiological activity of their parent neurons. The enkephalin-immunoreactive neurons, in distinction from the larger group of granule cells, may have moved into the enkephalin-synthesizing State-B as a consequence of physiological events occurring days earlier.

The indication from the seizure data that activity induces enkephalin synthesis may provide some interpretation for the ontogenetic data as well. As reviewed above, dynorphin and CCK are present within the hippocampal mossy fibers during the period of synaptogenesis and well before the morphological elaboration of the mossy fiber boutons. In contrast, enkephalin does not appear in this system until much later. By the size of the mossy fiber boutons first seen to contain enkephalin, one would estimate that this peptide does not appear until about a week after synaptogenesis. It is possible that enkephalin does not appear within the mossy fibers until the physiological activity of the granule cells or their afferents is sufficiently mature to induce synthesis.

The validity of the above suggestions is difficult to assess at the moment. Most importantly, we do not know whether nonepileptiform physiological activity influences the synthesis of enkephalin in the dentate gyrus granule cells. We do, however, have evidence for the colocalization of enkephalin and CCK in other mammalian brain areas (Gall et al. 1987) and for the independent regulation of colocalized enkephalin and CCK in invertebrates (Petrics-Gesser and Larsson 1985). There is also a growing body of evidence that colocalized peptides and classical neurotransmitters are differentially

and probably independently regulated by physiological activity in peripheral systems (Black 1984).

Returning from the cellular to the system level, the potential consequence of opposite fluctuations in enkephalin and CCK content in the mossy fiber system following seizures is very intriguing. CCK has been demonstrated to be antagonistic to endogenous-opioid-mediated processes in other systems (Faris et al. 1988). CCK has also been suggested to have anticonvulsant properties (Kádár et al. 1984) while enkephalin has been demonstrated to induce limbic seizures when injected into the hippocampus (Elazar et al. 1979). These factors lead one to speculate that the shift in CCK/enkephalin balance in the mossy fiber system following seizures may be of consequence to the future seizure susceptibility of the structure.

In closing, it might be appropriate to ask if the hippocampus has served, and continues to hold promise, as a fruitful model system for the study of opioid peptides in the brain. I suggest that the answer is yes, but in ways not anticipated 5 years ago. Although the specific contribution of the endogenous opioid peptides to synaptic physiology in the hippocampus and elsewhere remains largely unresolved, we have identified systems and paradigms with which we can study the physiological regulation of opioid gene expression in the brain, and possibly resolve mechanisms through which experience leaves its trace in the operation of neuronal circuitry.

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AUTHOR

Christine M. Gall, Ph.D.
Department of Anatomy and Neurobiology
University of California
Irvine, CA 92717

The Anatomical Relationship of Opioid Peptides and Opiate Receptors in the Hippocampi of Four Rodent Species

Miles Herkenham and Stafford McLean

INTRODUCTION

A commonly held assumption in neurobiology is that drugs and neurotransmitters bind to postsynaptic receptors located at sites of termination of central nervous system (CNS) pathways containing the relevant transmitter. One expectation, therefore, is that autoradiographic maps of receptor distributions should closely resemble (or match) the immunohistochemical maps of terminals of the related transmitters. This paper reports a test of that assumption in the rodent hippocampus, a cortical structure whose laminar organization has been well characterized for a number of neurochemicai systems, including opioid peptides and opiate receptors.

Currently, three families of opioid peptides have been identified: the proenkephalin gene yielding the enkephalins and other carboxy terminal extended forms, the prodynorphln gene providing the dynorphins, and the proopiomelanocortin gene providing beta-endorphin. Extensive work has documented enkephalin and dynorphin but not beta-endorphln immunoreactive systems in the rodent hippocampus.

The existence of three major opiate receptor subtypes in the brain is now accepted: the mu receptor exhibiting highest affinity for morphine and related alkaloids, the delta receptor binding enkephalin better than morphine, and the kappa receptor having a high affinity for ethytketocyclazocine and dynorphin (Lord et al. 1977; Chang et al. 1979; Chavkin et al. 1982; Corbett et al. 1982).

In this study, the distributions of mu, delta, and kappa opiate receptors were determined in the hippocampi of four species of rodent (squirrel, guinea pig, rat, and hamster) and compared with the distributions of immunoreactive dynorphln and enkephalin in each species. Although distributions of the peptides were fairly similar across species, the distributions of each of the receptor types showed marked species variations. Moreover, the terminal and receptor localization patterns did not typically correspond to each other, leading to the conclusion that different determinants underlie the distributions of each, After most explanations for these mismatches are considered and found lacking, it is proposed that transmitters like the opioid peptides act in a parasynaptic fashion to diffuse from release sites and bind to receptors on non-contiguous cells.

IMMUNOHISTOCHEMISTRY

The distribution of enkephalin and dynotphin in the anterior hippocampus is relatively invariant across the four rodent species. Most striking is the intense immunoreactivity in the mossy fiber system (figure 1 on pp. 36 and 37). In all species, mossy fibers immunoreactive for both peptides course from the dentate hilus into the stratum lucidum of the CA3 and CA2 regions. Here, the immunoreactivity is punctate and dense, indicative of a terminal zone.

in addition, isolated dynorphin-positive fibers are present in the stratum radiatum, and sparse enkephalin fibers are found throughout the CA fields. The dentate molecular layer contains occasional dynorphin- and enkephalin-positive processes which often can be traced back to immunoreactive granule cells.

At these rostral hippocampal levels, a dense plexus of enkephalin fibers in the hamster and rat straddles the hippocampal fissure, occupying superficial parts of both the dentate molecular layer and stratum lacunosum-moleculare of CA1 (figure 1). This immunoreactivity represents the enkephalinergic component of the perforant path, which originates in the entorhinal cortex (Fredens et al. 1984).

RECEPTOR AUTOGRAPHY

The distributions of the opiate receptor subtypes mu, delta, and kappa exhibit a variable relation to the distributions of endogenous

opioid peptides and considerable diversity among species. However, within a species, mu and kappa receptors are similarly distributed and, except for the rat, have a pattern which is generally the inverse of the delta pattern.

Squirrel

The dentate gyrus contains the greatest density of both mu and kappa receptors (figure 1). Another peak of dense mu and kappa receptors is found in the stratum lucidum of the CA3/CA2 fields. The pattern of delta receptors in the squirrel is strikingly different from the kappa and mu distributions. First, the stratum lucidum is notable for its lack of labelling. Second, delta sites are conspicuously absent from all cell layers. Third, in contrast to the low levels of mu and kappa receptors in the CA1 field, delta receptors are dense there.

Guinea Pig

In the guinea pig, the stratum lucidum has the densest kappa labelling. The distribution of mu receptors is similar to that of kappa receptors, but there are far fewer mu sites.

The delta binding pattern differs from the mu and kappa patterns. Dense binding is present in CA1 in all layers but the pyramidal cell layer and the superficial part of the stratum lacunosum-moleculare. Within the CA3 Reid, the region of the mossy fiber terminal zone has the lowest delta receptor density in the anterior hippocampus.

The dentate gyrus molecular layer is characterized by few delta receptors, though a band of higher density is found in its most superficial position, bordering the hippocampal fissure. The hilar region is moderately labelled and shows elevated binding subjacent to the granule layer.

Hamster

The hamster has kappa receptors densely and selectively distributed in the two locations that also contain intense peptide immunoreactivity, namely the dentate hilus and the stratum lucidum (figure 1). In addition, a moderate kappa receptor density fills the remaining dentate gyrus. The mu pattern is similar, but the density is much lower throughout. Mu and kappa binding is not detected

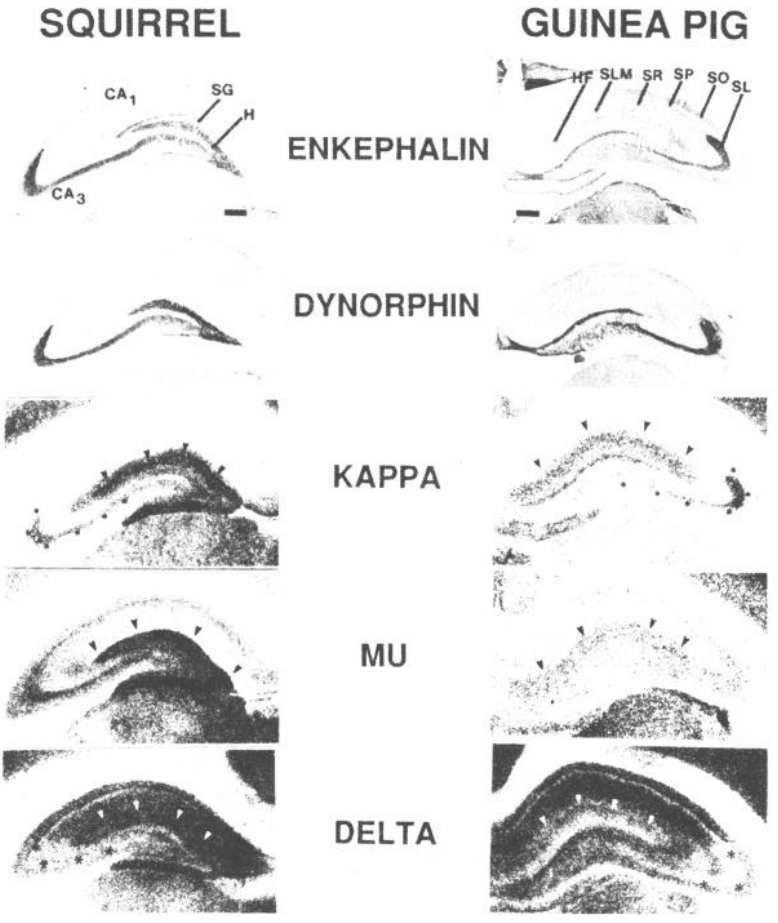
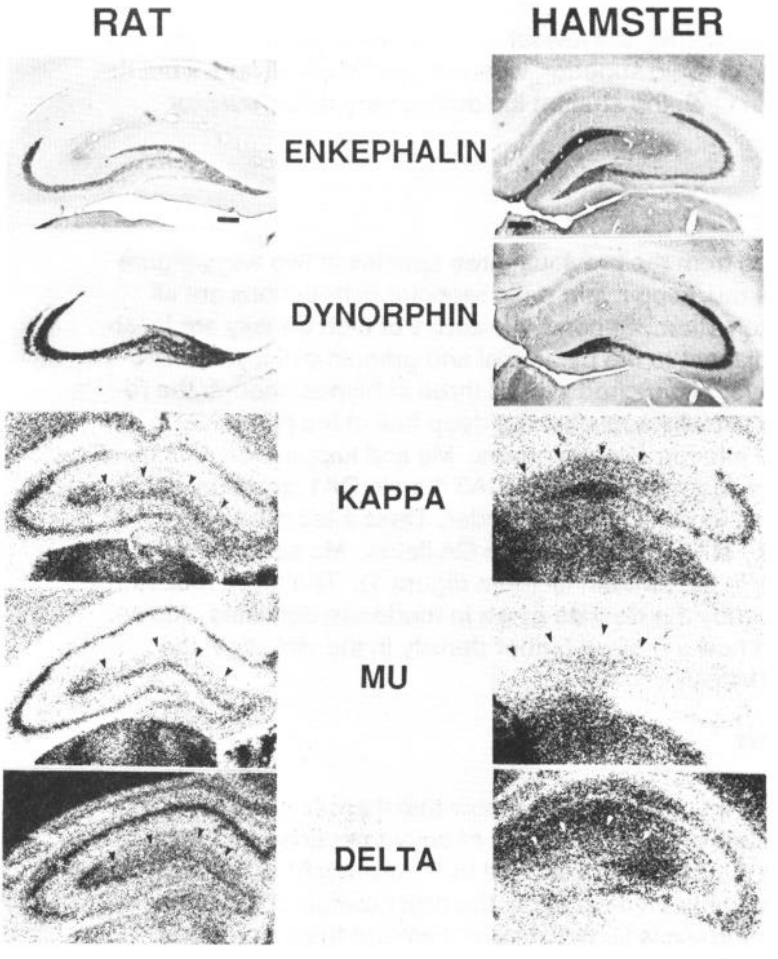


FIGURE 1. *Distribution of opioid peptides and receptors in the hippocampi of four rodent species*

NOTE: Immunoreactivity was performed using the avidin-biotin technique, and the receptor distributions were derived from autoradiographic film images. For immunohistochemistry, frozen and cut fixed sections were Incubated in antisera against dynorphin B (1:2,000) (E. Weber) or leu-enkephalin (1:1,000) (Immunonuclear). For receptor autoradiography, fresh frozen brains were cut and thaw-mounted onto slides. For mu binding, slides were incubated for 90 minutes at 25 °C in tris buffer with 2 nM [^{3}H]D-Ala^2, MePhe^4,Gly-ol^5 enkephalin. For delta binding, slides were Incubated in tris buffer and 30 nM oxymorphone with 4.8 nM



[^{3}H]O-Ala^2,D-Leu^5-enkephalin. For kappa binding, slides were transferred to MOPS buffer with 1 µM BIT and 1 µm FIT and incubated for 30 minutes at 25 °C. BIT and FIT are, respectively, selective mu and delta opiate receptor irreversible acylating agents (Rice et al. 1953). The sections were then incubated for 3 hours at 0 °C in phosphate buffer containing 0.4 M NaCl and 7 nM [^{3}H]bremazocine. Abbreviations: H, hilus; SG, stratum granulosum; HF, hippocampal Assure; SLM, stratum lacunosum-moleculare; SR, stratum radiatum; SP, stratum pyramidale; SO, stratum oriens; SL, stratum lucidum. Asterisks mark locations of SP. Arrowheads mark location of HF. Calibration bar measures 450 µM for squirrel and guinea pig and 250 µM for rat and hamster.

SOURCE: McLean et al. 1987.

in the CA1 field. In contrast, delta receptors show virtually the opposite pattern; they are lowest in the dentate gyrus and densest in hippocampal regio superior, where they occupy all layers but the pyramidal. In CA3, the stratum lucidum is very delta receptor sparse.

Rat

The rat differs from the previous three species in two ways (figure 1). First, the mu, kappa, and delta receptor distributions are all similar to each other. Second, the zones of high density are located in and adjacent to the pyramidal and granule cell layers. The pyramidal layer is enriched with all three subtypes, though the receptor band actually occupies the deep half of the pyramidal layer and extends into the stratum oriens. Mu and kappa receptors have a denser and wider band in CA2/CA3 than in CA1 , and they show an abrupt shift in density at the border. Delta sites display no major density shifts throughout the CA fields. Mu sites are selectively sparse in the stratum lucidum (figure 1). The three receptor subtypes occupy the dentate gyrus in moderate densities. Mu and kappa sites show a peak of higher density in the vicinity of the granule cell layer.

DISCUSSION

The results of the present study show that there is no obvious relationship between the locations of opioid peptides and opiate receptors in the hippocampus, and this “mismatch” is the main focus of the present study. In essence, the organization of the enkephalins and dynorphins is fairly consistent among the four rodent species examined, whereas the distribution of the opiate receptor subtypes is variable (figure 1). While both opioid and peptide terminals and opiate receptors display patterns that closely conform to the regional and laminar morphology of the hippocampal formation, the organizations of these pre- and postsynaptic elements are clearly different from each other.

This does not mean that very good correspondence between opioid peptides and receptors cannot be obtained. In the guinea pig and hamster, mu but especially kappa receptors are densely located in precisely the same locations (dentate hilus and stratum lucidum) that show intense opioid peptide immunoreactivity. Similarly, in the hamster and rat, the receptors in the outer third of the

dentate molecular layer and the adjacent stratum lacunosum-moleculare are aligned with enkephalinergic fibers, presumably terminals of the lateral perforant pathway. However, the dense distributions of kappa and delta receptors in the dentate molecular layers of the squirrel and guinea pig, respectively, are not matched by a corresponding density of peptides. Unlike the other rodents studied, the rat shows binding peaks for all three receptor subtypes in the regions of the cell body layers where peptides are not detected; conversely, kappa but especially mu receptors are practically absent in the peptide-rich mossy fiber terminal zone. Thus, individual instances of matches of peptides and receptors are present, but no consistent pattern of correspondence occurs across species. In fact, for all species examined, the delta receptors have unique patterns which typically show an inverse relationship with the pep tide densities and distributions.

Physiology

Administration of opiate alkaloids and peptides both *in vivo* (Nicoll et al. 1977; Chou and Wang 1977; Henriksen et al. 1982) and in slice preparations in vitro (Dunwiddie et al. 1980; Haas and Ryall 1980; Lee at al. 1980; Dingledine 1981) consistently produces increased hippocampal pyramidal cell activity. It has been concluded that the opiates, rather than acting directly on pyramidal cells, act instead to remove a tonic inhibitory input to the pyramidal cells via occupation of receptors on interneurons (Zieglgänsberger et al. 1979; Dunwiddie et al. 1980; Lee et al. 1980).

Within the CA1 field of the rat, microiontophoretic mapping studies have demonstrated that the stratum pyramidale and stratum oriens are sites most sensitive to the administration of enkephalins (Dingledine 1981). This is consistent with the elevated levels of all three opiate receptor subtypes in these layers.

Opiate receptor localization and opiate physiology create expectations about opioid peptide terminal locations, but these are not met by the relatively restricted localization of the peptides in the mossy fiber system, which is known to convey excitatory impulses to CA3 pyramidal cells, possibly using glutamate as the transmitter (Storm-Mathisen and Iversen 1979; Altschuler et al. 1985). In the stratum lucidum, mossy fibers which contain the highest density of opioid peptides in the hippocampus (Chavkin et al. 1985; Zamir et al. 1985) terminate selectively on pyramidal cell dendrites (Hamlyn

1962). Because opiates appear to exert their inhibitory effects on interneurons, opioid-containing processes should terminate on inter-' neurons rather than pyramidal cell dendrites. However, there is no evidence that any mossy fibers make selective inhibitory contacts with interneurons in the stratum lucidum of CA3. Thus, there is no currently known aspect of mossy fiber anatomy or physiology that fits with our knowledge of the major opiate actions in the hippocampus.

Mismatch Implications

The mismatches between opiate receptor locations and sites of release of opioid peptides at nerve terminals noted here for the hippocampus are part of a much larger and more general phenomenon that occurs for ail well-characterized neurotransmitter/receptor systems. We have pointed out (Herkenham and McLean 1988; Herkenham 1987) that there is a failure of correspondence, a mismatch, between the locations of nerve terminals and receptors throughout the central and even peripheral nervous system for all neuropeptides, monoamines (norepinephrine, dopamine, and serotonin), and "fast" transmitters (gamma-aminobutyric acid, acetylcholine, and glutamate). Explanations for the mismatches can be put into four categories:

(1) Typically, it has been assumed that mismatches represent technical failures and/or incomplete knowledge of the full endowment of transmitters or receptors within a family of chemically related substances.

(2) It has also been pointed out that we should not expect a match, since transmitters and receptors represent pre- and postsynaptic elements of different neurons which may express their features throughout their processes and not just at the sites of contact (Kuhar 1985).

(3) Biochemical possibilities for the discrepancies include occupied receptors (not available for binding by virtue of prebound endogenous ligand), spare receptors, and nonfunctional receptors.

(4) More recently, we have stressed the likelihood that many receptors, especially those to which the endogenous ligand binds with high affinity, are not located at synapses and may be

sites of action of transmitters released from a distance (Herkenham and McLean 1986; Herkenham 1987).

The present results can be used to test the various explanations for the mismatches. Addressing the issues in the first category, we have used the most sensitive and optimal histological techniques currently available and have provided quantitative analysis of the receptors using previously validated binding conditions. We have used markers that recognize the predominant forms of endogenous hippocampal opioid peptides and all of the major opiate receptor subtypes, so that it is unlikely that undiscovered ligands or receptors exist which would significantly alter the presently described relationships. Even if a low-affinity synaptic receptor is discovered that is selectively localized to terminal zones, we would still have to consider the roles of the more widely distributed and mismatched high-affinity sites.

Consideration of hippocampal anatomy sheds light on the second category. Given the horizontal stratification of the receptors and the vertical alignment of hippocampal dendrites, it is clear that, if receptors are localized on hippocampal neurons, then they are confined to restricted zones along the length of the dendritic tree. If some proportion of the receptors is located on afferent axons rather than on dendrites, then this population of receptors appears to be concentrated at axon terminals, as the major afferent fiber tracts do not show commensurate receptor densities. Thus, cells and fibers both show discrete receptor localization, making unlikely the suggestion that receptors in one hippocampal location are cytoplasmic and in transit to another location (Stengaard-Pedersen 1983), or that they reflect the ceil's inability to concentrate binding sites.

Transmitters, of course, are localized by immunocytochemistry throughout cell bodies, axons, and terminals, and this can be a basis for a mismatch if the sites of terminal release cannot be da termined. Opioid peptide immunohistochemistry indicates that the major hippocampal terminal zone lies in the stratum lucidum, whereas scattered fibers with varicosities suggest additional release sites in the dentate and CA fields. Another source of opioid-peptide-containing terminals is the perforant path (Gall et al. 1981; Stengaard-Pedersen et al. 1981; Tielen et al. 1982; Fredens et al. 1984), but this provides enkephalin fibers predominantly to more caudal (temporal) parts of the hippocampus rather than to the

rostral (septal) part examined in this study. Perforant path terminals are localized to the superficial parts of the hippocampai and dentate molecular layers. Thus, for the hippocampus, the major sites of peptide release are known, and these taken together are the basis for the present claim that there is no correlation between their locations and the distributions of the receptors.

Each of the various biochemical explanations for the mismatch in the third category can be put in perspective by the present results. The possibility of receptor occupation by the endogenous ligand is prevented by preincubating sections in buffer containing high concentrations of Na^+, which causes dissociation of endogenous agonists from the receptors (Garzón et al. 1984). A more compelling argument is that we show instances of dense mu and kappa receptors in the stratum lucidum, providing an excellent correspondence of dense receptors in a location where endogenous enkephalin and dynorphih are apparently released in high concentrations.

The possibility of spare receptors is an intriguing one that deserves further study. Spare receptors can be responsible for an amplification of biological response, which might be useful in locations where ligands exist in low concentrations. If spare receptors "explain" some of the hippocampal mismatches, then they must exist in high proportions in some hippocampal regions and not in others. As argued elsewhere for the substance P (Mantyh et al. 1984, Herkenham and McLean 1986; Herkenham 1987) and alpha-1-adrenergic systems (Johnson and Minneman 1985), regional correlations between receptor density and amplitude of biological response have not documented differential distributions of spare receptors. However, this line of inquiry could be fruitful in the opiate system.

Finally, within this third category, receptors are seen by some workers as either nonfunctional or "merely" drug-binding sites. This is an implausible view in light of the precision with which they are distributed, the metabolic expense and synthetic machinery required to maintain them, and the proven functionality in physiological studies showing responses to drug application localized in sites, such as CA1, where the concept of nonfunctional receptors might be most appealing because of a paucity of the endogenous ligand. In fact, within CA1 of the rat, the discrete distribution of opiate receptors in stratum pyramidale and adjacent stratum oriens

corresponds precisely with D-ala^2-D-leu^5-enkephalin (DADLE)-sensitive sites most effective in enhancing the population spike (Dingledine 1981).

The fourth category of explanations is centered around the possibility that many receptors are not localized to synapses. There is ample evidence for this hypothesis. In a recent electron micrographic autoradiography study, Hamel and Beaudet (1984) found that only a small percentage (7 percent) of mu opiate receptors in rat striatum are located at junctional contacts satisfying the usual criteria for synapses. Most sites were at nonjunctional, hence nonsynaptic, contacts between neuronal elements. It is well known that cell-to-ceil communication in the autonomic nervous system occurs without synapses (Merillees et al. 1983). Presynaptic modulation of neurotransmitter release by ehdogenous ligand in the CNS is likely to be nonsynaptic, insofar as electron micrographic studies almost never show axoaxonic synapses at nerve terminals. Diffusion of transmitter to distant sites has been documented in the peripheral nervous system (Jan and Jan 1983; Kravitz et al. 1983).

The possibility that nonsynaptically located opiate receptors are targets of opioid peptides released from distant sites may shed light on a fundamental question: Why does the CNS utilize so many opioid peptides and receptor subtypes? One hypothesis, promoted by several laboratories and based on relative affinities and potencies in bioassays, is that a given peptide is targeted to a particular type of opiate receptor. An alternative hypothesis is that many forms of opioid peptides can interact with each of the receptor subtypes: thus, source and availability of ligand, ligand stability, receptor microenvironment, as well as kinetics of the receptor/ligand interaction may play important roles in intercellular information transfer.

The cerebrospinal fluid (CSF) is a possible conduit for diffusion of endogenous opioid peptides to targets, such as the hippocampus, that are close to the ventricles. Ventricular administration of beta-endorphin causes hippocampal seizure activity by diffusion of this endogenous ligand into the hippocampal neuropil (Henriksen et al. 1978). Seizure activity itself causes release of an endogenous opioid material into the CSF, and this material diffuses to distant neuronal sites to alter the threshold for subsequent seizures (Tortella and Long 1985). The sources and nature of the endogenous neurochemicais and the precise targets for the opiate effects

are currently unknown, and speculation about the role that hippocampal opiate receptors might play in generation or inhibition of seizures is beyond the scope of the present data. However, we can speculate that dense high-affinity opiate receptors in the hippocampus may be localized to participate in cell-to-cell communication transmitted nonsynaptically at very low ligand concentrations. This mechanism is consistent with proposed roles of opiates as neuromodulators of pyramidal cell activity.

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AUTHORS

Miles Herkenham, Ph.D.
Stafford McLean, Ph.D.

Unit on Functional Neuroanatomy
National Institute of Mental Health
Building 36, Room 2D-10
Bethesda, MD 20892

Seizure-Induced Alterations in the Metabolism of Hippocampal Opioid Peptides Suggest Opioid Modulation of Seizure-Related Behaviors

Jau-Shyo Hong, Jacqueline F. McGinty, Laura Grimes, Tomoyuki Kanamatsu, Johnny Obie, and Clifford L. Mitchell

INTRODUCTION

Endogenous opioid peptides appear to play an important role in the regulation of brain excitability, especially in the seizure-sensitive hippocampus. Proenkephalinderived peptides have a unique excitatory action when iontophoretically applied to pyramidal cells of the hippocampus, whereas in most other brain areas these peptides manifest inhibitory actions (Nicoil et al. 1977). Electroencephalographic (EEG) recordings have indicated that intracerebroventricular (ICV) or intrahippocampal injections of enkephalins or beta-endorphin have either proconvulsant (Elazar et al. 1979; Henriksen et al. 1978; Urca et al. 1977) or anticonvulsant (Tortella and Cowan 1982) properties depending on experimental conditions (Frenk 1983). Unlike proenkephalin- or proopiomelanocortin-derived peptides, however, prodynorphin-related peptides have a more complex electrophysiological profile. Intracerebroventricular dynorphin does not induce epiieptiform activity in the rat, and single-unit and field potential studies show mixed effects on CA3 neuronal excitability with more inhibitory responses than are seen with the enkephalins (Henriksen et al. 1982; Walker et al. 1982; Gruol et al. 1983; Henriksen et al., this volume; Chavkin et al., this volume). Sub cutaneous or ICV injection of the specific kappa agonist U50 488 provides protection against certain electroconvulsive shock (ECS)-induced seizures in rats, suggesting an anticonvulsant role of endogenous kappa agonists such as dynorphin (Tortella et al. 1986).

Seizure activity exerts profound effects on the metabolism of opioid peptides in certain seizure-sensitive brain regions such as the hippocampus, amygdala, and substantia nigra. We have employed

different models of seizures, such as ECS (Hong et al. 1979; Yoshikawa et al. 1985; Kanamatsu et al. 1988a), amygdaloid kindling (McGinty et al. 1988), or kainic acid (Hong et al. 1980; Kanamatsu et al. 1986b), to study the interrelationship between brain opioid peptides and seizures. It is the purpose of this chapter to review our progress in this area.

ELECTROCONVULSIVE SHOCK

Effects of Repeated ECS on the Metabolism of Enkephalins

It has been suggested that endogeneous opioid peptides may mediate some ECS-elicited behavioral alterations, including analgesia, retrograde amnesia, changes in seizure threshold, and postictal depression, since these behaviors can be prevented or altered by pretreatment with opiate antagonists (Carrasco et al. 1982; Holaday and Belenky 1980; Tortella and Cowan 1982; Urca et al. 1981; Urca et al. 1988). The increase in met^5-enkephalin-like immunoreactivity (ME-LI) in certain limbic areas of the rat brain, such as the septum, amygdala, nucleus accumbens, hypothalamus, and hippocampus (figure 1 and table 1), produced by repeated ECS treatment, also suggests a role for ME in the behaviors listed above. In contrast, beta-endorphin content in the hypothalamus was not altered by repeated ECS, suggesting specific ME involvement (Hong et al. 1979; Kanamatsu et al. 1986a). In order to determine whether the increase in ME-LI elicited by repeated ECS was due to an increase in the biosynthetic activity of enkephalin-containing neurons, we measured the abundance of messenger RNA (mRNA) coding for preproenkephalin A, using both in vitro cell-free translation and blot hybridization using a complementary DNA (cDNA) clone coding for preproenkephalin A derived from rat brain. The results demonstrated that repeated ECS increased the abundance of mRNA coding for preproenkephalin A in the hypothalamus (Yoshikawa et al. 1985) and hippocampus (Li et al., in preparation). This finding suggests that ECS increases the metabolic rate of ME-LI and also may trigger a large release of this opioid peptide from nerve terminals. This possibility was further supported by our recent report that repeated ECS downregulates the opioid receptors in rat brain (Nakata et al. 1985). Ten consecutive daily ECS treatments caused a reduction of mu and delta receptor binding in the hypothalamus, hippocampus, and caudate nucleus, but not in the frontal cortex and brain stem. These changes of opioid receptor binding were not observed in rats receiving a single ECS. Scatchard analysis revealed

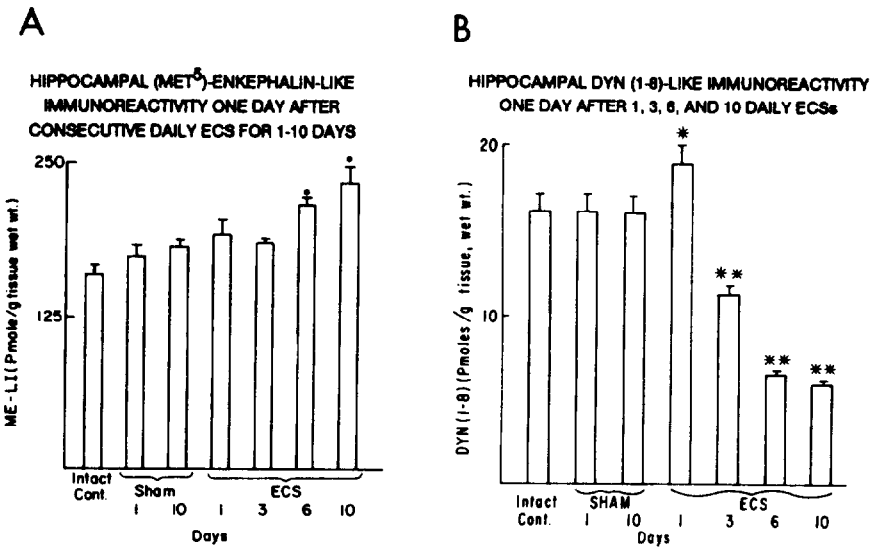

FIGURE 1. *Hippocampal met[5]-enkephalin and dynorphin A (1-8) levels 1 day after 1 to 10 daily ECS treatments*

NOTE: (A) Met[5]-enkephalin levels were significantly increased 1 day after 6 or 10 daily ECS treatments ($p<0.05$). (B) Dynotphin A levels were significantly decreased 1 day after 3, 6. or 10 ECS treatments ($p<0.01$).

SOURCE: Kanamatsu et al. 1986a. Copyright 1986, Society for Neuroscience.

that ECS-induced reduction of opioid receptor binding was due to a decrease in the binding but not to a change in the binding affinity. Similar to the changes in ME-LI, time-course studies showed that, 7 days after the end of 10 consecutive ECS treatments, both mu and delta receptor binding remained lower than those of sham controls, but the effect disappeared in 2 to 3 weeks. These observations are consistent with the hypothesis that ECS treatments increase the release and biosynthesis of opioid peptides in certain brain regions, which in turn downregulates the opioid receptors (Nakata et al. 1985).

TABLE 1. *ECS-induced changes in hippocampal opioid peptide levels*

ECS Treatment	Dynorphin A	Met^5-enkephalin
5 Minutes After:		
1 ECS	-40%	±0%
1 Day After:		
1 ECS	+10%	±0%
3 ECS	-30%	±0%
6-10 ECS	-70%	+40%
2 Weeks After:		
6-10 ECS	±0%	±0%

Effects of Repeated ECS on the Metabolism of Dynorphins

We have found that the metabolism of prodynorphin-derived peptides is also very responsive to repeated ECS treatments in different brain regions (Kanamatsu et al. 1986a). Ten daily ECS treatments caused a significant increase in dynorphin A (1-8)-like immunoreactivity (DN-LI) in most limbic-basal ganglia structures including the hypothalamus, striatum, and septum. These results are very similar to those for ME-LI. However, the ECS treatments decreased DN-LI in the hippocampus by 64 percent, while ME-LI increased 40 percent (figures 1 and 2 and table 1). A possible anatomical basis for this was revealed by immunocytochemistry, which showed that dynorphin A (1-8) immunostaining was depleted in the mossy fiber pathway, and enkephalin immunostaining was increased in the perforant pathway. The differential responsiveness of enkephalin- and dynorphin-containing neurons in the hippocampus can be observed in other seizure models including kainic acid and amygdaloid kindling (see below). These observations suggest that different mechanisms may regulate these two opioid peptide systems in the hippocampus, and that they play different

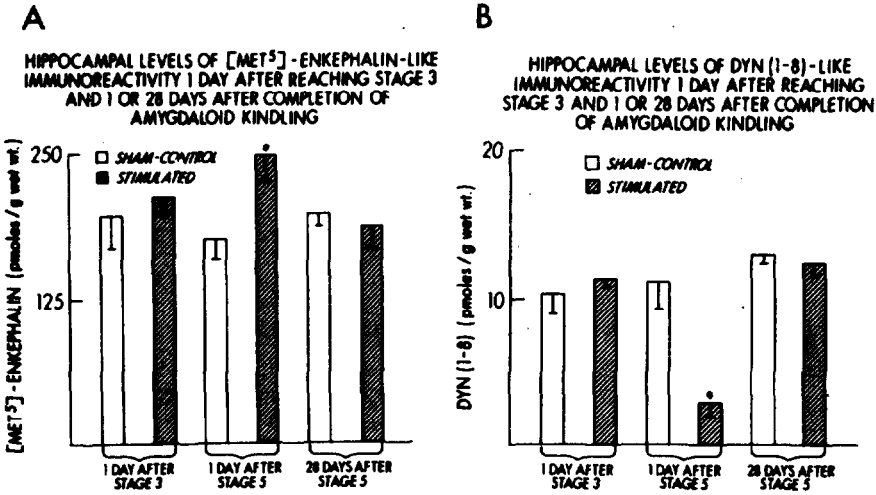

FIGURE 2. *Hippocampal opioid peptide levels 1 day and 28 days after amygdaloid kindled seizures*

NOTE: (A) ME-LI is increased significantly ($p<0.05$) 1 day after reaching two consecutive stage 5 seizures only. (B) DN-LI is decreased significantly ($p<0.001$) 1 day after reaching stage 5 seizures only.

SOURCE: McGinty et al. 1986, Copyright 1986, Elsevier Scientific Publishers Ireland Ltd.

roles in mediating hippocampal excitability by interacting with different receptor subtypes.

Effects of a Single ECS on the Metabolism of Enkephalins and Dynorphins

Recently, we examined the effects of a single ECS on brain opioid peptides. Time-course studies revealed that, within 15 minutes after a single ECS, there was a 40 percent decrease of DN-LI in the hippocampus, but no change was found in the other brain regions examined, including the hypothalamus, striatum, and brain stem (Takeuchi et al., in preparation). Furthermore, levels of ME-LI, substance P, and cholecystokinin (CCK) (1-8) were not altered in the hippocampus, suggesting that hippocampal dynorphin-containing neurons are most sensitive to ECS, and changes in

dynorphin metabolism are most correlated with ECS-elicited changes in seizure threshold or retrograde amnesia.

KINDLING-INDUCED SEIZURES

Effects of Amygdaloid Kindling on the Metabolism of Enkephalins and Dynorphins

Kindling is an experimental model of temporal lobe epilepsy in which initially subthreshold stimulations result in progressively more intense seizure activity (stages 1 to 4), until a generalized (stage 5) seizure occurs (Goddard 1967; Racine 1972). Recent studies have suggested that two dynorphin-rich structures, the hippocampal formation and the substantia nigra, are involved in the development of kindling and expression of a fully kindled seizure, respectively (McNamara et al. 1966). There is, however, no evidence that alterations in opioids or other neurotransmitters or receptors are responsible for the permanence of the kindling phenomenon (Woodbury 1984).

Several lines of evidence have indicated involvement of endogenous opioid peptides in modulating amygdaloid-kindled seizures. Naloxone, a stereoselective opioid antagonist, has been reported to facilitate amygdaloid kindling in rats (Hardy et al. 1980). In addition, the duration of postictal depression can be altered by opioid agonists and antagonists (Frenk et al. 1979). Enkephalin has been reported to be increased in whole rat brain (Vindrola et al. 1981), and dynorphin has been observed to be increased in rabbit hippocampus 1 day after reaching stage 5 seizures (Przewlocki et al. 1983). In order to construct a time course of opioid peptide changes during and after amygdaloid kindling, we examined rats 1 day after reaching subconvulsive stage 3 seizures, and 1 day and 4 weeks after two consecutive stage 5 seizures (table 2). One day after stage 3 seizures, no significant change in opioid peptide levels was observed by radioimmunoassay (RIA) or immunocytochemistry (ICC). In contrast, 1 day after two consecutive stage 5 seizures, there was a 50 percent increase in ME-LI in the projection from the entorhinal cortex to hippocampal pyramidal cells (the temporoammonic component of the perforant pathway) and a 70 percent decrease in DN-LI in the mossy fibers of the hippocampus (McGinty et al. 1986). The only other change was an increase in the

TABLE 2. *Kindling-induced changes in hippocampal opioid peptides*

Site	Dynorphin A	Met^5-enkephalin	
Amygdala			
1 Day After:			
Stage 3	±0%	±0%	
Stage 5	-71%	+43%	
28 Days After:			
Stage 5	20%	±0%	
Prepiriform			
		HPC	ERC
5 Minutes After			
Stage 2	±0%	+34%*	+167%***
Stage 5	-23%	+30%*	+237%***
1 Day After:			
Stage 2	+13%	+41%**	+221%***
Stage 5	-12%	±0%	+84%*
6 Weeks After:			
Stage 2	±0%	±0%	±0%
Stage 5	±0%	±0%	±0%

*p<0.05
**p<0.01
***p<0.001

NOTE: HPC=hippocampus: ERC=entorhinal cortex.

immunostaining of dynorphin in the substantia nigra pars reticulata, a structure implicated in the regulation of kindled seizure thresholds (McNamara et al. 1984). Normal levels of both peptides in the hippocampus and substantia nigra returned by 4 weeks after completion of kindling. A recent study by Naranjo et al. (1986) indicates that the increase in the levels of hippocampal enkephalin after amygdaloid kindling is due to an increase in the biosynthesis

of this peptide. Therefore, while opioid peptides are not involved in the permanence of the kindling phenomenon, kindling-induced seizures stimulate different changes in the metabolism of dynorphin and enkephalin, suggesting separate regulation of each peptide in states of hyperexcitation.

Effects of Prepiriform Cortex Kindling on the Metabolism of Enkephalin and Dynorphin

In order to further examine the role of opioid peptides In the development and expression of seizures, we have determined the levels of opioid peptides in different brain regions kindled by stimulation of the deep prepiriform cortex (DPC) once every hour until stage 2 or stage 5 seizures occurred. The DPC has recently been identified as a critical epileptogenic site in the brain (Piredda and Gale 1965). In contrast to the large decrease in hippocampal dynorphin levels 1 day after reaching stage 5 amygdaloidkindled seizures, DN-LI was slightly but significantly elevated in DPG kindled hippocampus 5 minutes after stage 2 seizures, and slightly depressed 5 minutes after stage 5 seizures (table 2). However, there was a large increase in ME-LI in the entorhinal cortex (ERC) and a moderate increase in the hippocampus (table 2) among other areas 5 minutes or 24 hours after reaching stage 2 seizures, and 5 minutes after two consecutive stage 5 seizures (Zhao et al., in preparation). Twenty-four hours after reaching stage 5 seizures, the ME-LI increase in the ERC had moderated, and the elevation in the hippocampus had disappeared. These data suggest that the development of prepiriform-kindled seizures has more of an effect on enkephalin in the ERC than on dynorphin in the hippocampus. These data are supported by the observation that an ICV injection of beta-fulnaltrexamine (a long-lasting mu receptor antagonist), reduced the number of DPC stimulations required to reach stage 5 seizures by about 50 percent (Lee et al., unpublished observations). These observations are consistent with previous reports which promote an anticonvulsant role of opioid peptides in electrically stimulated seizures (Hardy et al. 1980; Frenk et al. 1979).

KAINIC-ACID-INDUCED SEIZURES

Kainic acid (KA) is an excitatory amino acid which causes hippocampal epileptiform activity and motor seizures. When applied intracerebrally, it is most toxic to hippocampal pyramidal cells in fields CA3 and CA4 (Nadler et al. 1978), which are innervated by

enkephalin- and dynorphincontaining mossy fibers. Several laboratories have employed this neurotoxin to provide a model for human temporal lobe epilepsy (Ben-Ari 1985; Nadler 1981). Administration of KA to rats by various routes elicits a repertoire of epileptiform behaviors that depends upon the dose and the particular route of administration. Systemic injection and injection into the amygdala both produce a syndrome consisting mostly of wet dog shakes (WDS) followed by gnawing, sniffing, and masticatory movements, followed by repetitive head bobbing, rearing, forelimb clonus, and sudden loss of upright posture (Ben-Ari et al. 1980). Hippocampal injections of KA produce WDS, bilateral proptosis, forelimb clonus, and episodes of hyperactivity characterized by circling away from the injected side (Schwartz et al. 1978). Lower doses of intraventricular KA cause WDS, but higher doses of KA produce tonic-clonic seizures (Lanthorn and Isaacson 1978). Usually, the seizures last 3 to 6 hours depending on the doses used.

Effects of KA on the Metabolism of Hippocampal Enkephalins and Dynorphins

We have employed KA as a tool to perturb the metabolism of opioid peptides in the hippocampus so we could study in detail the molecular mechanisms regulating the biosynthesis, processing, and release of these peptides. Initially we found that, 2 days after a single intraventricular or intrastriatal dose of KA, the level of ME-LI increased significantly in the hippocampus and remained elevated for 2 weeks, as demonstrated by RIA (Hong et al. 1980). Subsequently, we found that leu^5-enkephalin-like immunoreactivity detected by RIA and ICC was increased in mossy fibers and perforant path fibers, whereas the level of DN-LI was unchanged in mossy fibers 4 days after KA administration (McGinty et al. 1983). These alterations were our first indication that the metabolism of dynorphin and enkephalin could be differently regulated, not only in the same brain structure but in the same pathway.

Recently, we assayed hippocampal opioid peptides by RIA and ICC at early time points after a single intrastriatal injection of KA (Kanamatsu et al. 1986b; figure 2 and table 3). Three to six hours after KA administration, when recurrent seizures were still apparent, DN-LI in mossy fibers was depleted 70 percent, and ME-LI in mossy fibers and in the perforant path was depleted by 30 to 40 percent. By 24 to 48 hours, the levels of both peptides had risen above normal, with DN-LI peaking at 48 hours at 171 percent of

normal, and ME-LI at 270 percent of normal. However, by 72 hours, ME-LI levels had risen to 300 percent of normal, especially in the mossy fibers, while DN-LI in the mossy fibers had returned to normal. The reduction in DN-LI and ME-LI within 3 to 6 hours of KA administration suggests an increase in the release of these peptides during the seizure period.

TABLE 3. *Kainic-acid-induced changes in hippocampal opioid peptide levels*

Kainic Acid (Intrastriatal)	Dynorphin A	Met^5-enkephalin
3 hours	-40%	-30%
8 hours	-70%	-40%
24 hours	+35%	+95%
48 hours	+65%	+170%
72 hours	±0%	+200%

To determine whether the increase in hippocampal ME-U elicited by KA reflected changes in ME biosynthesis, levels of mRNA coding for preproenkephalin ($mRNA^{enk}$) and cryptic ME-LI cleaved by enzyme digestion from preproenkephalin were measured (figure 3). Following the convulsant period (8 hours), $mRNA^{enk}$ was 400 percent of control, and by 24 hours, cryptic ME-LI was 300 percent of control. Increases in native and cryptic ME-LI and $mRNA^{enk}$ were also noted in the entorhinal cortex but not in the hypothalamus or uninjected strlatum. Our data suggest that KA-induced seizures cause an increase in ME release, followed by a compensatory increase in ME biosynthesis in the hippocampus and entorhinal cortex.

The finding that hippocampal $mRNA^{enk}$ was increased following KA administration is consistent with our previous finding that protein synthesis inhibition by cycloheximide attenuated the late increase in hippocampal ME levels of KA-treated animals without affecting ME levels of controls (Hong et al. 1980). Work is under way in our laboratory to determine the effects of KA on preprodynorphin mRNA biosynthesis. It is likely that our mRNA studies will indicate an increase in KA-induced dynorphin biosynthesis because

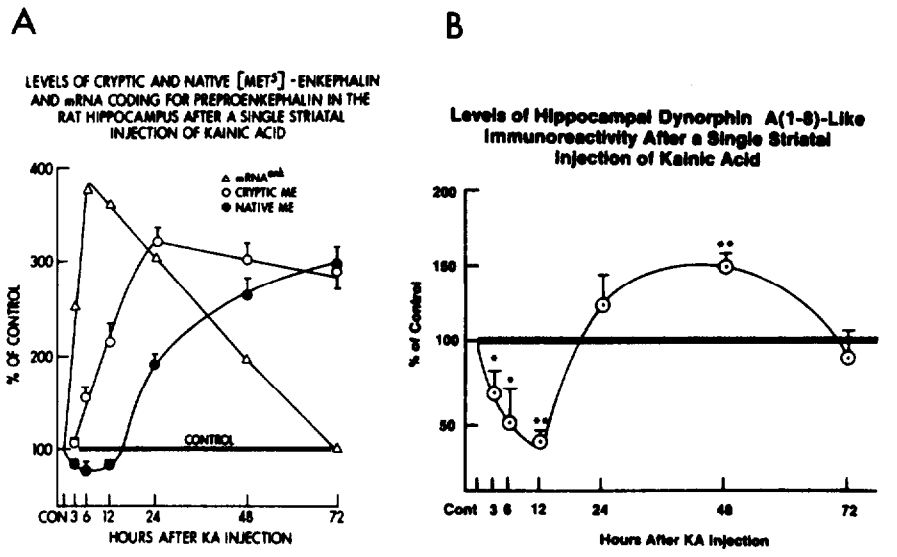

FIGURE 3. *Hippocampal opioid peptide and mRNA levels after intrastriatal kainic acid administration*

NOTE: (A) Levels of cryptic and native met^5-enkephalin and mRNA coding for preproenkephalin 3 to 72 hours after kainic add. (B) Levels of dynorphin A (1-8) 3 to 72 hours after kainic add.

SOURCE: Kanamatsu et al. 1986b, Copyright 1986, Society for Neuroscience.

cycloheximide, coadminlstered with intra-amygdaloid KA, has been shown to block the late increase in hippocampal dynorphin immunoreactivity, indicating that proteln synthesis is necessary for the effect to occur (Larson et al. 1963).

Possible Roles of Hippocampal Opioid Peptides in KA-Induced WDS

Several studies indicate that release of enkephalins from neurons either within or afferent to the hippocampal formation is critical to KA-induced WDS. ICV injections of enkephalin (Leybink et al. 1978; Wei et al. 1977; Drust and Connor 1983), beta-endorphln (Bloom et al. 1976), or kainic acid (Lanthorn and Isaacson 1978) cause naloxone-reversible WDS, suggesting that both agents produce WDS by actions at opiate receptors. Electrolytic lesions of the CA3 but not the CA1 region of the hippocampus attenuate WDS produced by ICV injections of KA (Isaacson and Lanthorn 1981),

suggesting that WDS-producing agents act on CA3 neurons rather than on their CA1 targets. In view of the release of both enkephalin and dynorphin when hippocampal mossy fibers are activated (Chavkin et al. 1983), we have carded out a series of experiments to distinguish the possible roles of opioid peptides In the expression of WDS (Grimes et al., in press).

First, we have confirmed that pretreatment with naloxone attenuates KA-elicited WDS. We injected into rats 2.5, 5.0, or 10.0 mg/kg of naloxone intraperitoneally. Five mg/kg of naloxone reduced the number of KA-induced WDS by approximately 50 percent.

To determine which opioid peptide participates in KA-induced WDS, we injected ICV antisera against either met^5-enkephalin or dynorphin A (1-8) 4 minutes before KA was administered subcutaneously. Antisera against met^5-enkephalln, but not dynorphin A (1-8), significantly attenuated WDS. These data indicate that enkephalin, but not dynorphin, may be associated with KA-induced shaking behavior.

The cellular basis for KA- and opioid-induced WDS was explored by injecting D-ala^2-D-leu^5-enkephalin (DADLE) and dynorphin A (1-8) into ventral or dorsal hippocampus. Ten µg of DADLE injected into ventral, but not dorsal, hippocampus elicited 50 to 100 WDS within a 20-minute period, whereas the same dose of dynorphin elicited less than 10 WDS.

Finally, colchicine injections into ventral hippocampus abolished KA-induced WDS but not seizures. We injected colchicine, a cytotoxin which selectively destroys dentate granule cells, into dorsal and/or ventral hippocampus to determine which part of the hippocampus is important for the expression of KA-induced WDS. Colchicine caused equivalent decreases (81 percent) of ME-LI in dorsal and ventral hippocampus; however, the concentration of ME-Li was nearly four times greater in ventral than in dorsal hippocampus. Therefore, colchicine caused a greater absolute ME-LI decrease in the ventral than in the dorsal region of this structure. Immunocytochemical and histological data showed that the enkephalin decrease was largely attributable to a destruction of dentate granule cells and the concomitant reduction in staining of the mossy fiber axons.

Following KA administration, WDS were not observed in rats with colchicine-induced lesions of the ventral hippocampus. Lesions restricted to the dorsal hippocampus caused a nonsignificant attenuation of KA-induced WDS. These studies suggest that the ventral hippocampus is an important site for KA-induced WDS. The far greater concentration of enkephalin in ventral than in dorsal hippocampus offers an explanation of why wlchicine lesions of this region were sufficient to eliminate KA-induced WDS, and why the action of anti-met^5-enkephalin was most effective in reducing KA-induced WDS in the ventral hippocampus.

In contrast to the effects on WDS, none of the wlchicine lesions had effects on KA-induced behaviors such as facial and forelimb clonus, rearing, and hypersalivation. These findings do not rule out the possibility that enkephalin plays a role in hippocampal seizures, but they do suggest that enkephalin released from mossy fibers is not essential for seizure expression.

SUMMARY

The evidence accumulated so far indicates that seizure activity exerts profound changes on the metabolism of opioid peptides in the hippocampus. Our data consistently show a large transient decrease in dynorphin and a modest decrease in enkephalin in the hippocampus following either a single ECS or KA injection. These initial reductions, which are indicative of increased release, may trigger the biosynthetic process of hippocampal opioids and result in an overproduction of the peptides seen in the rebound phase. However, the amount and timing of the rebound in enkephalin and dynorphin levels in response to repeated ECS, amygdaloid kindling, or KA differ drastically: a rapid and sustained increase in ME-LI follows all three treatments, in contrast to a slow recovery after a large and sustained decrease in DN-LI induced by repeated ECS and amygdalold kindling. These results, which are unique to the hippocampus, suggest that differential mechanisms are operative in regulating the metabolism of these two opioid peptides in the hippocampus. It is likely that a well-coordinated regulation of hippocampal function can be achieved through the differential release of enkephalin and dynorphin and their subsequent interactions at different subtypes of opioid receptors following seizure activities.

From a functional point of view, our data provide a neurochemical correlate of previous reports that brain opioid peptides may mediate ECS-induced behavioral alterations, such as changes in seizure threshold, postictal depression, and retrograde amnesia. The robust changes in the levels of opioid peptides in kindled rats, plus shortening of the kindling process by pretreatment with mu opioid antagonists, strongly suggest the involvement of brain opioid peptides in the development of kindling. Finally, these studies show clear evidence that enkephalin in the hippocampus is important in KA-induced WDS, a component of the opiate withdrawal syndrome in rodents (Isaacson and Lanthom 1981). Further studies should help distinguish the regulatory mechanisms responsible for changes in opioid peptide metabolism during states of hyperexcitability in the hippocampai formation.

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AUTHORS

Jau-Shyong Hong, Ph.D.
Laboratory of Behavioral and Neurological Toxicology
National Institute of Environmental Health Sciences
P.O. Box 12233
Research Triangle Park, NC 27709

Jacqueline F. McGinty, Ph.D.
Department of Anatomy
East Carolina University School of Medicine
Greenville, NC 27858

Laura Grimes, Ph.D.
Curriculum in Toxicology
University of North Carolina at Chapel Hill
Chapel Hill, NC 27514

Tomoyuki Kanamatsu, Ph.D.
Department of Physiology
Toho University
Tokyo, Japan

Johnny Obie, B.S.
Laboratory of Behavioral and Neurological Toxicology
National Institute of Environmental Health Sciences
P.O. Box 12233
Research Triangle Park, NC 27709

Clifford L. Mitchell, Ph.D.
Laboratory of Behavioral and
Neurological Toxicology
National Institute of Environmental
Health Sciences
P.O. Box 12233
Research Triangle Park, NC 27709

Opioids in the Hippocampus: Progress Obtained From In Vivo Electrophysiological Analyses

Steven J. Henriksen, James B. Wiesner, and Guy Chouvet

INTRODUCTION

The earliest evidence implicating a role for endogenous opioids in hippocampal function derived from a survey of the actions of electrophoretically applied opioid peptides on various central nervous system (CNS) structures (Nicoll et al. 1977). The unique excitatory actions of opioid peptides on the spontaneous discharge of presumed hippocampal pyramidal neurons, when compared to inhibition of neuronal discharge observed for most other brain areas, provided the impetus for an increasingly detailed analysis of the actions of opioids in this structure (see Henderson (1983), Corrigall (1983), and Siggins and Gruol (1986) for reviews). In parallel studies, potential clinical relevance of hippocampal opioids was provided by a series of investigations conducted in unrestrained, freely moving animals. Similar studies conducted in several laboratories demonstrated that minute doses of intracerebroventricularly or intracerebrally administered opioid peptides elicited nonconvulsive epileptiform seizures in rats (Urca et al. 1977; Henriksen et al. 1978; Tortella et al. 1978; Elazar et al. 1979; Cain and Corcoran 1984). Significantly, these electrographic seizures were found to have their origin in limbic structures, the major apparent focus being the hippocampus (Henriksen et al. 1978; Henriksen et al. 1983b), and could be elicited with doses of opioids that were devoid of analgesic or other overt behavioral signs (Henriksen et al. 1978; Frenk 1983). In the past 5 years, with more precise localization of hippocampal opioids and their receptor subtypes (McGinty, this volume; Gall, this volume; Herkenham and McLean, this volume), a systematic and more relevant functional analysis of hippocampal opioids has been possible. The following essay will review the

critical hippocampal studies employing *in vivo* methodologies and will summarize the most recent evidence concerning the potential roles opioids may play in hippocampal function and pathology.

ENCEPHALOGRAPHIC INVESTIGATIONS

As with the study of many other candidate neurotransmitters in the CNS, electrophysiological observations of opioid effects on neurons preceded precise immunohistochemical localization of hlppocampal opioids and/or their receptors. Early studies employing intracerebroventricular injections of the opioid peptides beta-endorphin and leucine-enkephalin suggested a proconvulsant action for these substances. Figure 1 illustrates an example of the onset of such an event following beta-endorphin administration in a rat.

The multichannel encephalographic record shows the abrupt initiation of high-voltage paroxysmal discharges recorded from numerous sites. Systematic bipolar electrode recordings of subcortical brain loci strongly suggested, however, that the hippocampus and amygdala were the primary origins of this activity (Henriksen et al. 1978). Subsequent investigations using carbon-14-labelled 2-deoxyglucose ([^{14}C]2-DG) in conjunction with intracerebroventricular beta-endorphin injections demonstrated conclusively that the brain areas showing major increases in 2-DG uptake following beta-endorphin coincided with the sites where the greatest electrical abnormalities were recorded (figure 2).

In contrast, most other brain areas examined showed significant decreases in 2-DG uptake during endorphin-induced seizures. These early studies suggested that opioid peptides could produce pathological alterations in limbic neural activity and that the hippocampus could play a major role in the elaboration of these epileptiform events. In an attempt to generalize these results to other species, similar experiments employing a variety of other animals have been conducted using beta-endorphin (Henriksen and Ehlers, unpublished results). Intracerebroventricular injection of opioid peptides to squirrel monkeys, cats, or rabbits have failed to evoke the type of paroxysmal electrical discharge observed in rats (figure 3).

Although intermittent isolated high-voltage sharp waves were frequently elicited, nothing akin to the frank ictal seizure episodes were observed, even at dose levels producing behavioral analgesia

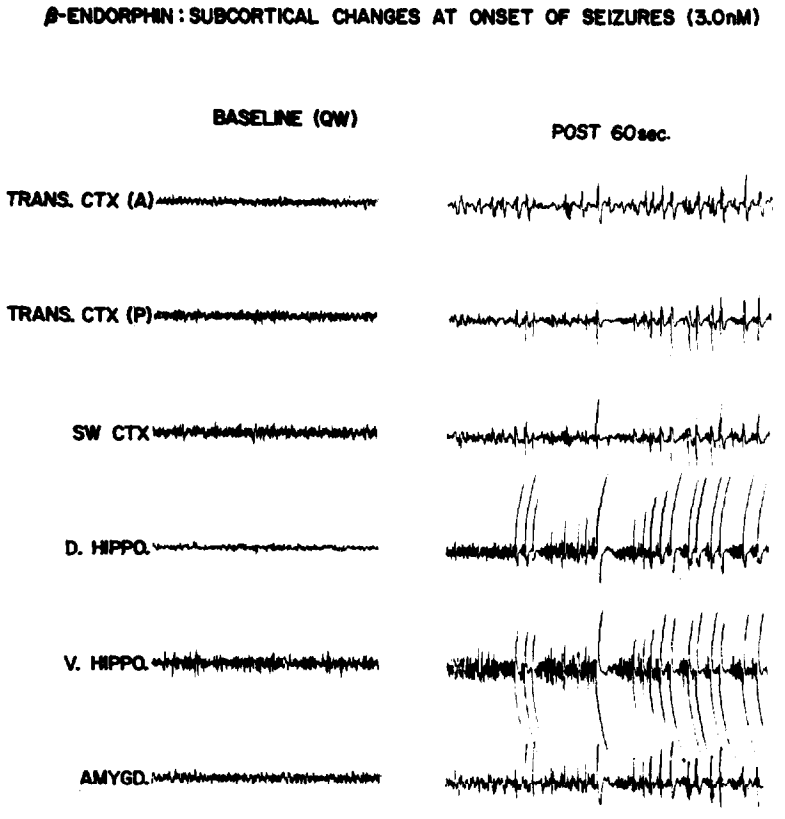

FIGURE 1. *Multitrace EEG record of beta-endorphin-induced epileptiform activity in the rat*

NOTE: Beta-endorphin was administered intracerebroventricularly in a total dose of 3 nM at the end of the baseline trace. The top three traces are bipolar recordings of cortical EEG; the fast three traces are bipolar recordings taken from deep electrodes in dorsal and ventral hippocampus.

and catatonia. In fact, in certain animal species exhibiting spontaneous or stimulus-induced seizures, opioid peptides and certain opiate alkaloids have proved to be anticonvulsant (Meldrum et al. 1979; Bajorek and Lomax 1982). Moreover, more recent studies in rat have demonstrated pro- as well as anticonvulsant properties of opioid peptides depending on the seizure model employed and the specificity of the ligand for particular opioid receptor subtypes (Tortella et al. 1981; Tortella et al. 1985; Tortella et al. 1987;

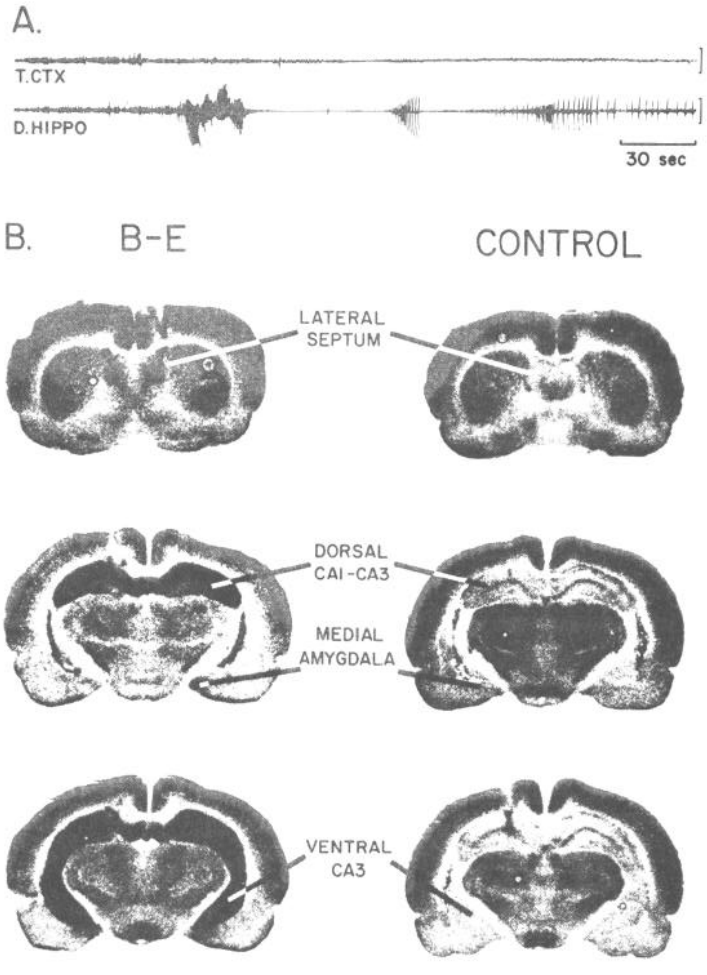

FIGURE 2. *EEG traces and [^{14}C]2-DG autoradiographic micrographs of the response to intracerebroventricularly administered beta-endorphin (3 nM) in a representative rat*

NOTE: (A) Electrographic tracing of ictal seizures episode elicited by beta-endorphin (B-E). (B) [^{14}C]2-DG autoradiographs of representative sections of control rat brain and the brain of a rat exhibiting seizure shown in figure 2A following B-E.

Henriksen et al. 1978; Snead and Beardon 1980; Frenk 1983). The role of hippocampal opioids in seizure activity has been further complicated by the observation that spontaneous and artificially

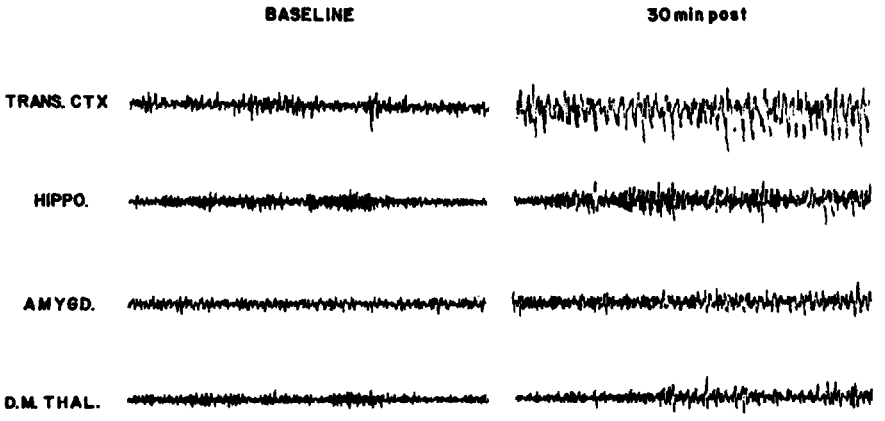

FIGURE 3. *Intracerebroventricular beta-endorphin in the squirrel monkey*

NOTE: EEG traces recorded from cortical and subcortical sites following the administration of 200 µg of beta-endorphin intracerebroventricularly. Note high-voltage slow cortical EEG associated with fast hippocampal rhythms.

induced seizure episodes in experimental animals are associated with changes in the postseizure content of opioids in this structure (Hong et al. 1980; McGinty et al. 1983; Hong et al., this volume; Gall, this volume). Finally, postictal behavioral as well as electrographic activity in several seizure models have been shown to be sensitive to naloxone, indicating that endogenous opioids potentially released during seizures may serve to decrease the probability of subsequent seizures and the aberrant postictal and/or interictal behaviors (Frenk et al. 1979; Caldecott-Hazard et al. 1984; Bajorek et al. 1986).

To summarize, a decade of data obtained using electroencephalographic tools has suggested a multitude of intriguing relationships. First, it appears that ventricularly administered opioids can produce naloxone-reversible synchronized epileptiform events in rat hippocampi, and at least some paroxysmal activity in other species as well. This indicates that opioids can act at receptors in the hippocampus to facilitate specific patterns of excitation/inhibition at the cellular level which result in the complex EEG waveforms described above. Second, hippocampal opioids may also function to

attenuate and/or block the recurrence of ictal episodes by causing a selective depression of cellular excitability. Last, the specific opioid receptor subtypes, the specific distribution of endogenous opioid ligands, and the localization of opioids within the intrinsic circuitry of the various subfields of the hippocampal formation may be the critical factors underlying the observed EEG actions of opioids and their antagonists.

CELLULAR INVESTIGATIONS

Early *in vivo* extracellular studies of opioid effects on hippocampal neurons were consistent with the EEG studies described above. That is, electrophoretic application of endorphin peptides elicited increases in spontaneous discharge of isolated hippocampal neurons in contrast to the decreases in activity observed in other brain areas (Hill et al. 1977; Nicoll et al. 1977; Zieglgänsberger et al. 1979). In addition, prolonged ejection of beta-endorphin from adjacent micropipettes elicited paroxysmal unfiltered potentials that

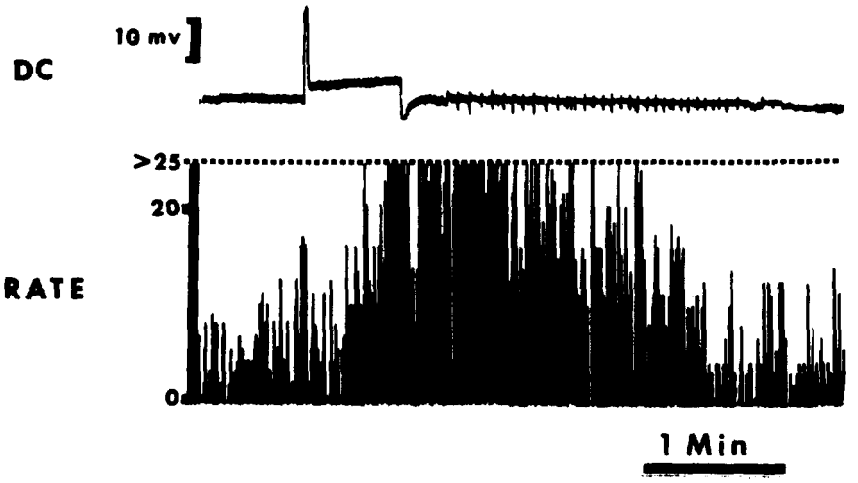

FIGURE 4. *Effects of electrophoretically applied beta-endorphin on hippocampal CA 1 neuron discharge and local EEG*

NOTE: Upper trace: DC unfiltered recording (low gain) from microelectrode tip. Beta-endorphin (150 nA) ejected during DC deflections. (Note sharp waves emerging following beta-endorphin application.) Bottom trace: Ratemeter record of individual CA1 neuron. (Note increase in spontaneous rate associated with beta-endorphin application.)

were synchronous with EEG-recorded epileptifom activity (French and Siggins 1980) (figure 4). These data provided the first cellular evidence substantiating the excitatory action of opioids on hippocampal neurons and demonstrated that this global effect could occur as a direct result of the local application of opioid peptide, rather than being mediated indirectly through distant, extra-hippocampal processes.

These cellular electrophysiological findings were made in the absence of firm evidence of either a significant concentration of opiate receptors (identified by the use of tritiated naloxone), or of substantial amounts of opioid peptide in the hippocampus. However, with the demonstration first of enkephalin-like immunoreactivity in the hippocampus (Gall et al. 1981) and, later, of dynorphin-like immunoreactivity (McGinty et al. 1983; McGinty 1985), with a clear difference in the distribution of these two peptides in the hippocampus, an impetus was provided to investigate the potential roles of the endogenous opioids in those hippocampal areas of preferred opioid distribution (Gall, this volume; McGinty, this volume).

Early immunohlstochemical investigations of endogenous oploids in rodents demonstrated broad distribution in most limbic structures but sparse labelling of cells in the hippocampus (Sar et al. 1978; Bloom and McGinty 1981). More recent radioimmunoassay studies, however, have revealed a significant concentration of leu^5-enkephalin (ENK)-immunoreactivity (IR) in the dentate-CA3 fields with only half as much ENK-IR in CA1 and the subiculum (Bayon et al. 1983).

The mossy fiber pathway originating in dentate granule cells and projecting through the hilus to innervate CA3 pyramidal cell dendrites was originally thought also to contain primarily enkephalin (Gall et al. 1981). The enkephalin antisera used in these earlier immunochemical studies, however, also cross-reacted with dynotphin. Subsequent histochemical studies using more specific antisera (McGinty et al. 1983) have shown that mossy fibers primarily possess immunoreactivity for dynorphin(s) and a much lower level of proenkephalin-derived opioids (Khachaturian et al. 1983; McGinty et al. 1984, White et al. 1987). This pathway was previously thought to be exclusively excitatory, releasing an acidic amino acid such as glutamate or aspartate. The presence of prodynorphin-derived peptides and an excitatory amino acid in the same pathway has aroused speculation that these substances, and perhaps others

(Gall, this volume), may be cotransmitters within the same mossy fiber terminals.

In addition, more recent hlstochemical studies of the rat CA1 hippocampal field demonstrate scattered dynorphin-IR and enkephalin-IR cells and fibers (Bayon et al. 1983; Gall et al. 1981; McGinty et al. 1984). Enkephalin-IR and proenkephalin-IR (but not dynorphin-IR) are also observed in the rat entorhinal cortex in cells that project to the hippocampus and dentate gyrus (McGinty 1985). Thus, opioid immunoreactivity in the three opioid peptidergic systems of the hippocampal formation represents the presence of three different prohormonal systems: (1) predominantly prodynorphin-derived peptides in the dentate-CA3 mossy fiber pathway; (2) predominantly proenkephalinderlved peptides in scattered interneurons in CA1 and CA3; and (3) proenkephalinderived peptides in the entorhinal to dentate gyrus perforant pathways.

The localization of the opioid peptides in nerve cells and fibers of the hippocampus suggest that they are likely transmitter candidates. This possibility is supported by recent biochemical studies showing that at least five related prodynorphin/neoendorphin derived peptides can be released from hippocampal slices by high K^+ in a Ca^2+dependent manner (Chavkin et al. 1983a). These include dynorphin A(1-17), dynorphin A(1-8), dynorphin B, alpha-neoendorphin, and beta-neoendorphin. Proenkephalin derived peptides are also released in this preparation (Chavkin et al. 1983a; Chavkin et al. 1983b). In light of acknowledged distributional differences in endogenous hippocampal opioids, it is of heuristic value to discuss separately the three major hippocampal cellular fields-CA1, CA3, and dentate gyrus-and their respective responses to exogenously applied opioids.

NEUROPHARMACOLOGY OF OPIODS IN AREA CA1

Due primarily to their dorsal location in the hippocampal formation and resultant ease in isolation and recording stability, cells in the CA1 field were early targets for analysis of opioid effects. In CA1, enkephalin immunoreactlvity is distributed as a band of reactive vaticosities within the border of the stratum radiatum and stratum lacunosum-moleculare.

When opioids, including the enkephalins beta-endorphin and dynorphin A, are ejected from micropipettes in the vicinity of CA1

pyramidal neurons *in vivo,* the consensus effect is to increase the spontaneous discharge of these cells (see Henderson (1988) and Siggins and Gruol (1986) for reviews). These effects are naloxone reversible and therefore presumed to be mediated by opiold receptors. As previously demonstrated (see figure 4), large Iontophoretic currents of beta-endorphin during single-unit recording of CA1 neurons can trigger repetitive large DC potentials suggestive of epileptiform activity (French and Siggins 1980). The single-unit excitation and epileptiform activity are intrinsic to the hippocampus, since they can also be evoked by superfusion of opioids into intraocular transplants of hippocampus (Taylor et al. 1979).

Considerable effort has been expended to analyze and comprehend the mechanisms underlying the paradoxical excitatory effects of opioids on hippocampal neurons (see Siggins and Gruol (1988) for a review). *In vivo* studies of hippocampal neurons using gamma-aminobutyric acid (GABA) blockade by bicuculline and blockade of transmitter release by Mg^{2+} ions indicate that the excitatory

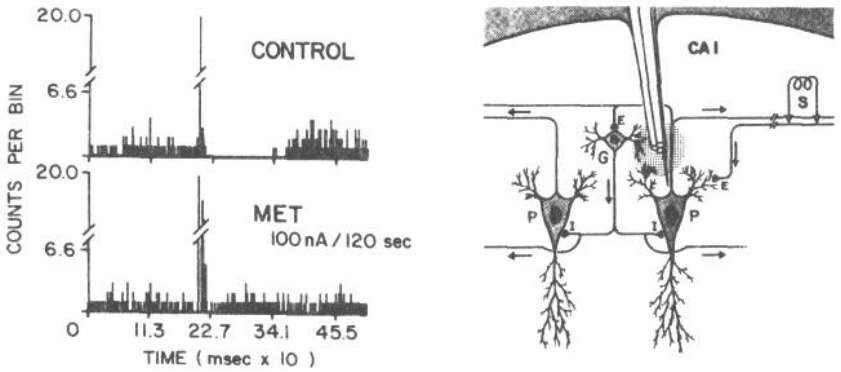

FIGURE 5. *A model for opioid disinhibition*

NOTE: Left panel: Poststimulus histograms before and during electrophoretic application of met-5-enkephalin on a CA1 neuron. During control, antidromic activation is followed by a prolonged inhibitory sequence. This inhibition (mediated by GABA interneurons) is blocked by met-5-enkephalin electrophoresis. Right panel: Proposed model of disinhibition (see text).

responses of hippocampal CA1 pyramidal neurons may be viewed as indirect, resulting from a primary inhibitory effect of the opioid on neighboring inhibitory interneurons, leading to excitation of pyramidal cells by dlsinhibitlon (Zieglgänsberger et al. 1979; Lee et al. 1980). Figure 5 illustrates the proposed model for this interaction. Despite this evidence, extracellular studies cannot reveal the exact mechanisms behind this unique opioid excitation. Several laboratories have therefore conducted intracellular studies using *in vitro* preparations such as hippocampal slices or explant cultures.

Such intracellular studies of pyramidal cells of the hippocampus CA1 have shown little or no direct effects on membrane potential or input resistance of morphine or several opioid peptides (leu^5-enkephalin; met-5-enkephalin; D-ala^2,D-leu^5 enkephalin; D-ala^2,D-leu^5-enkephalin amide; beta-endorphin) in concentrations up to 50 µM (Dingledlne 1981; Gahwiler and Herding 1981; Gruol et al. 1988; Haas and Ryall 1980). The lack of a direct membrane effect on pyramidal cells is predicted by the disinhibition hypothesis. Unfortunately, there has not been complete agreement as to the mechanism by which the opioids alter synaptic potentials. Intracellularly, most studies on the hippocampal slice indicate that the enkephalins and beta-endorphin primarily reduce the size of presumed GABAergic recurrent and feedforward inhibitory postsynaptic potentials (IPSPs) in both the CA1 and CA3 fields, and perhaps in the dentate gyrus as well (Gruol et al. 1988; Masukawa and Prince 1982; Nicoll et al. 1980; Siggins and Zieglgänsberger 1981). This also supports the hypothesis of a disinhibltory mechanism. Disinhibltion appears to be exerted presynaptically to the pyramidal cell since pyramidal cell responses to GABA, the likely transmitter for these IPSPs, are not reduced by the opioids (Dingledine 1981; Nicoll 1982).

However, in other hippocampal slice studies (Dingledine 1981; Haas and Ryall 1980), only enhanced excitatory postsynaptic potentials (EPSPs) or, conversely, a reduction of EPSPs (as well as IPSPs and depolarizing glutamate responses) were observed in about one-half of the pyramidal neurons studied (Siggins and Zieglgänsberger 1981).

It is still uncertain whether the observed hippocampal opioid effects are due to direct hyperpolarization of interneurons, to a true modulation of transmitter release, or to a more remote action. However, the bulk of the extracellular data (Dunwiddie et al. 1980; Lee et al. 1980; Nicoll 1982; Zieglgänsberger et al. 1979) and

intracellular data (see Robinson and Deadwyler (I 981) and text above) still support a disinhibitory mechanism of action for the single-unit excitatory and epileptigenic effect of these opioid peptides in the hippocampus (see Corrigall (1983) for a review). Still, facilitation of excitatory transmission has not been ruled out entirely. Some discrepancies between laboratories might be ascribed to (1) different methods of afferent stimulation *in vitro* and difficulties in selectively stimulating pure excitatory or inhibitory pathways; (2) the use of different opioid agonists acting on different opiate receptors; (3) the method of drug administration; and/or (4) the fact that the hippocampal pyramidal cell is usually the subject of these intracellular recordings, while the primary site of opioid action appears to be presynaptic to this cell. However, a preliminary intracellular study by Nicoll and Madison (1984) demonstrated that enkephalin analogues hyperpolarize hippocampal interneurons by increasing their potassium conductance, thus directly supporting the disinhibiiion hypothesis. in another recent *in vitro* extracellular investigation, Raggenbass et al. (1985) studied the action of a variety of opioids selective for certain opioid receptor subtypes on nonpyramidal neurons in the CA1 region. These authors found that the mu-selective agonist [D-Ala^2-MePhe^4-Gly-ol^5-enkephalin (DAGO), but not the kappa-selective agonist U50,488H, decreased the spontaneous discharge of presumed inhibitory interneurons in a naloxone-reversible fashion. These cellular data support earlier field-potential studies (see below) suggesting that the indirect, excitatory action of opioids in all hippocampal cell fields studied may be mediated by a mu- and perhaps a delta-opioid receptor (Chavkin et al. 1985; Chavkin et al., this

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Another important electrophysiological tool used successfully to assess the effects of opioids in the hippocampus is the measurement of cellular excitability following afferent pathway stimulation. Because of the precise laminar arrangement of neurons, dendrites, and axons in the hippocampus, large synaptic potentials can be recorded with electrodes situated in selected strata of the hippocampal formation. These potentials are believed to represent local synchronous synaptic input evoked by afferent stimulation (Anderson et al. 1971). Although few studies have investigated the effects of opioids on these stimulated potentials *in vivo,* the existing data support the proposition that opioids elicit disinhibitory effects when tested in this assay system. Early studies by Linseman and Corrigall (1982) demonstrated that systemic morphine

potentiated the population field potentials evoked by stimulation of the afferent pathways to both CA1 and the dentate gyrus in the freely moving rat. Dunwiddie et al. (1980) described similar results for d-met-5-enkephalin amide in the CA1 region in urethane-anesthetized rats and, further, demonstrated that the action of the opioid could be mimicked by the GABA antagonist picrotoxin. In addition to the *in vivo* studies described above, numerous *in vitro* investigations have examined the effects of opioids in the CA1 area of the hippocampal slice preparation (Dingledine 1981; Dunwiddie et al. 1980; Gawiler and Herding 1981; Gruol et al. 1983; Haas and Ryall 1980; Masukawa and Prince 1982; Nicoll et al. 1980; Roblnson and Deadwyler 1981; Siggins and Zieglgänsberger 1981; and many more). In reviewing these data, two flndings emerge as wmmon denominators. First, superfusion or local application of opioid peptides increases the excitability of neurons in CA1 as documented by an increase in the evoked population spike and field EPSP. Second, opioids frequently give rise to multiple afterpotentials, which follow the primary population spike and are likely to be the result of the opioid-mediated decrease in recurrent inhibition (see Corrigall (1985) for a review).

In an attempt to define more precisely the opioid receptor type mediating these effects on evoked field potentials, two pharmacological strategies have been employed. First, several investigators have used highly selective opioid receptor agonists to determine the specificity of opioid action in the hippocampus (Iwama et al. 1986; Vidal et al. 1984; Wiesner and Henriksen 1987a; Raggenbass et al. 1985; Chavkin et al., this volume). Second, Chavkin et al. (1985) employed selective receptor inactivation in conjunction with selective opioid agonists to further characterize the opioid receptor sub types mediating the alteration in cellular excitability. Roth lines of investigation have revealed that, throughout the hippocampus, the opioid-induced increase in cellular responsiveness is mediated by both mu and delta opioid receptors. The kappa-selective opiates U50,488H and ethylketocyclazocine are inactive when tested in physiologically meaningful dose ranges (Chavkin et al., this volume).

In summary, the findings from *in vivo* investigations of opioid neuropharmacology in the CA1 region of the hippocampus are consistent with the pharmacological actions of opioids observed in both freely moving animals and *in vitro* preparations. These effects are also consistent (Dingledine 1981) with the observed anatomical distribution of opioids in this hippocampal subfield (Gall, this volume;

McGinty, this volume) as well as the regional distributions of opioid receptor subtypes as assessed by ligand-binding studies (Herkenkam and McLean, this volume). The main effect of exogenously applied opioids is to decrease tonic and recurrent inhibitory processes in presumed GABAergic interneurons, leading to an increase in spontaneous discharge rate of individual CA1 pyramidal neurons and an increase in neuronal excitability assessed by afferent stimulation. Despite the relative unanimity regarding the major pharmacologic actions of opioids in CA1, other questions regarding the role of opioids in hippocampal function remain: (I) There is no known neurophysiological event altered by the systemic or local administration of the opioid antagonist naloxone (but see Gallagher (this volume)); (2) controversy still attends the explanation of the cellular mechanism underlying disinhibition in CAI; and (3) substantial quantitative differences are seen in opioid-induced effects from *in vivo* compared to the *in vitro* preparation. These unresolved issues, not discussed in detail here, suggest that the hippocampal slice preparation may have severely compromised intrinsic inhibitory processes that may in turn taint opioid responsiveness (Dunwiddie et al. 1980; lwama et al. 1986; Durand et al. 1981), and/or anesthetics employed by neuropharmacologists *in vivo* may alter opioid neuropharmacology or cellular responsiveness.

NEUROPHARMACOLOGY OF OPIOIDS IN AREA CA3

Far fewer studies of opioids in the hippocampus have been directed toward pyramidal cells in subfield CA3. CA3 neurons, however, provide a unique assay for opioid function in the hippocampus because, in addition to scattered enkephalinergic terminal and cell bodies located in the zone stratum radiatum, CA3 neurons receive a massive projection of prodynorphinderived peptides carried by the mossy fibers of the dentate granule cells (Gall et al. 1981; McGinty et al. 1983; Gall, this volume; McGinty, this volume). CA3 neurons therefore have the potential for being acted upon by endogenous opioids indirectly through the mechanism described for subfield CA1 , as well as directly through prodynorphinderived peptides released from the mossy fiber terminals. Results from *in vivo* studies of CA3 neuron responsiveness to exogenously applied opioids reflect these complexities. Met-^{5}-enkephalin and its stable analogues uniformly increased the spontaneous discharge of CA3 neurons in *in vivo* electrophoretic experiments (Henriksen et al. 1982a; Henriksen et al. 1983a; Moises and Walker 1985). These effects were naloxone reversible and similar to the responses seen for cells in the CA1

region of the hippocampus. They likely reflect the same disinhibitory processes proposed for those neurons (see above). In contrast, prodynorphinderived opioids have less consistent effects when applied to CA3 neurons *in vivo.* Henriksen et al. investigated the effects of several prodynorphin-derived peptides (dynorphin A(1-17), dynorphin B, alpha-neoendorphin, dynorphin A(1-8), and des-tyr-dynorphin A(2-17)) on spontaneous discharge of CA3 neurons as well as on cellular excitability (Henriksen et al. 1982a; Henriksen et al. 1983a; Henriksen et al. 1983b). We observed that, in halothane-anesthetized rats, dynorphin A(1-17), dynorphin A(1-8), and dynorphin B, but not dynorphin A(2-17), had predominantly excitatory (77 percent) effects on CA3 neurons. These effects were naloxone reversible.

Figure 6 illustrates the prolonged excitatory effect of electrophoretically applied prodynorphin products on a CA3 neuron. Given the results obtained in CA1, where mu agonist activity for dynorphin A(1-17) has been observed *in vitro* (Chavkin et al. 1985), and the fact that the effect in CA3 can be blocked by the synaptic uncoupling agent magnesium, the excitatory effects of dynotphin on CA3 neurons probably reflects a disinhibiiory action of the peptide similar to that observed for enkephalin in both cell fields. However, variable percentages of CA3 neurons respond to dynorphin (and other kappa-receptor-selective opiates) with a decrease in

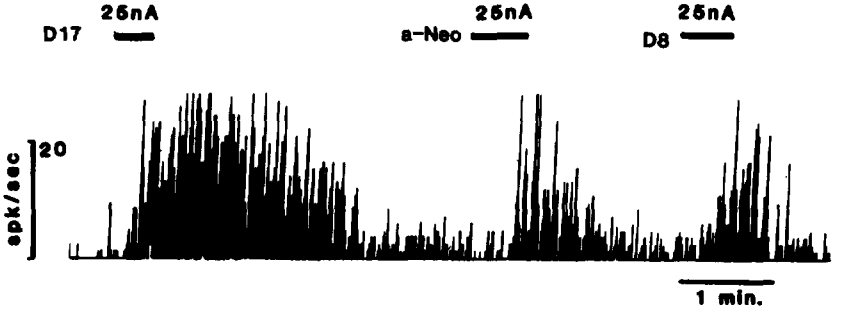

FIGURE 6. *Excitatory effects of prodynorphin-derived peptides on a CA3 neuron*

NOTE: Electrophoretic application of dynorphin A(1-17), alpha-neoendorphin. and dynorphin A(1-8). Note apparent relative potencies.

spontaneous discharge (Brookes and Bradley 1984, Moises and Walker 1985; Henriksen et al. 1982a; Henrlksen et al. 1983b).

Figure 7 is an example of dynotphin- and ethylketocyclazocine (Win 38-197)-induced inhibltion of a CA3 neuron with partial naloxone reversal. It appears therefore that the direct effect of dynorphin on CA3 neurons may be inhibitory, consonant with the direct actions of opioid peptides in many other brain areas. Moreover, the more selective the drug is for kappa opioid receptors, the higher the percentage of inhibition of CA3 neuron one obtains, suggesting that the observed inhibitory effects may be mediated by kappa receptors. Figure 8 is an example of inhibition of a CA3 neuron elicited by the highly selective kappa ligand, U50,488H.

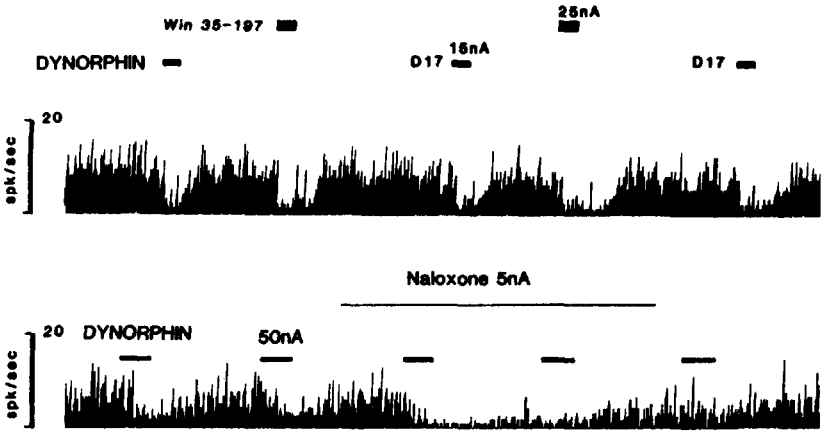

FIGURE 7. *Inhibitory response to dynorphin*

NOTE: Top: Ratemeter records from a CA3 hippocampal neuron during electrophoretk administration of dynorphin (dyn A(1-17)) and ethylketocyclazocine (Win 35-197). Bottom: Naloxone antagonism of dynorphin-induced inhibition. Note that, even with low ejection current (5 nA) of naloxone, there is a substantial decrease in the apontaneous firing rate, between the ejectlng pulses, underlying a possible nonspecific effect for naloxone.

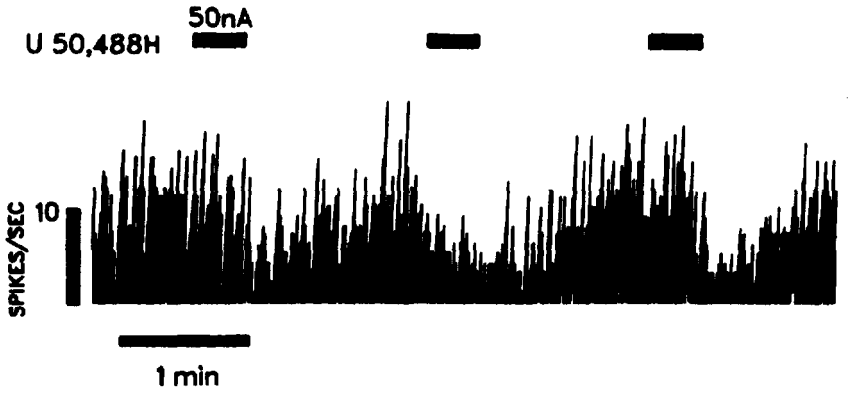

FIGURE 8. *Ratemeter record of a CA3 neuron during iontophoretic applications of kappa agonist U50,488H*

NOTE: Note the somewhat higher than usual spontaneous firing rate, whkh permits observation of U50,488H Inhibition.

SOURCE: Siggins et al. 1986, Copyright 1982, Raven Press.

Preliminary intracellular studies of dynorphin effects on CA3 pyramidal neurons in the hippocampal slice are relatively consistent with these extracellular findings: depending on the particular cell study, either slow hyperpolarizing or depolarizing responses are seen with low concentrations of dynorphin A and dynorphin B (Gruol et al. 1983). The changes in membrane potential were occasionally associated with changes in discharge activii in a direction opposite to that expected (Gruol et al. 1983), thus raising the possibility of an indirect action of dynorphin. The depolarizing responses were also not of the type expected of mossy fiber activation, being slow and not always capable of activating spikes. Similar mixed or weak effects in CA3 were seen *in vitro* with respect to the action of dynorphins on extracellular field potentials evoked by mossy fiber stimulation, although inhibitory action was sometimes observed (also see Iwama et al. (1986)).

Preliminary studies in our laboratory on the effect of locally applied dynorphin A(1-17) or naloxone on mossy fiber evoked field potentials *in vivo* have yielded other intriguing results. Using pairs of stimulus pulses to assess the action of opioids on sequential

recurrent inhibition and/or potentials, we have observed that neither dynorphin nor naloxone has effects on either the primary or secondary evoked population spike. The second evoked response, however, is followed occasionally by an afterpotential. This afterpotential is potentiated by electrophoretically applied dynorphin A(1-17) and inhibited by electrophoretically applied naloxone (see figure 9).

These results imply that an opioid peptide released by mossy fiber stimulation, although not altering the primary excitatory effects on CA3 neurons, may serve as a modulatory agent enhancing s&sequent inputs to the CA3 area in a frequency-dependent fashion.

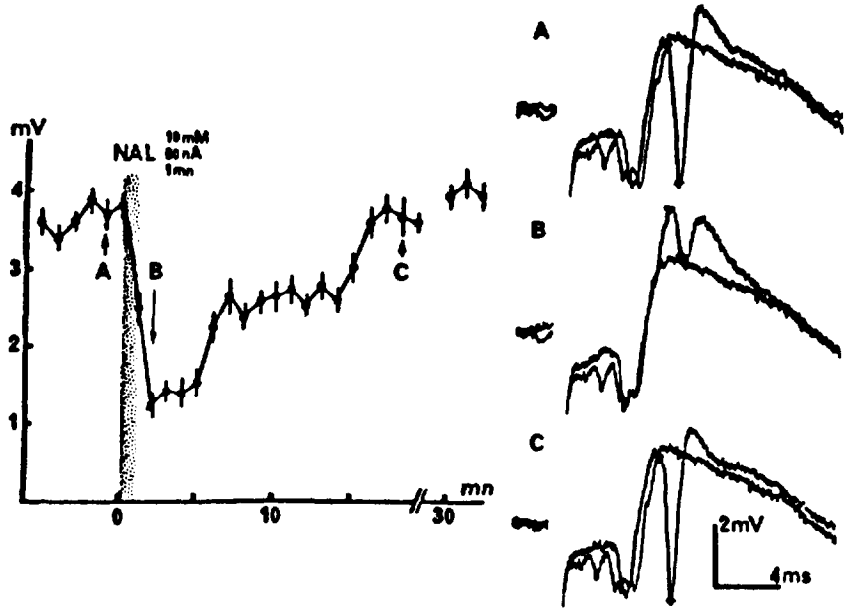

FIGURE 9. *Effect of iontophoretically applied naloxone on CA3 field potentials evoked by mossy fiber stimulation*

NOTE: Field potentials in (A) represent CA3 overlaid double pulse stimulation responses (interstimulus interval was 185 msec). Note emergence of potential at approximately 8 msec during second stimulus. In (B), note decrease In this potential. In (C), recovery follows naloxone electrophoresis. Response is plotted at left and represents the mean of 10 responses at each point.

NEUROPHARMACOLOGY OF OPIODS IN THE DENTATE GYRUS

As demonstrated by immunocytochemical studies (Gall et al. 1981; McGinty et al. 1983), the fascia dentata of the hippocampus contains endogenous opioid peptides from various anatomical sources. Three known sources of opioids observred in the dentate gyrus are (1) an enkephalinergic pathway arising from the entorhinal cortex; (2) intrinsic enkephalinergic local circuit neurons; and (3) the dentate granule cells themselves, which contain predynorphin-derived peptides (Christian et al. 1985; Linseman and Corrigall 1982; Wiesner et al. 1988; Wiesner and Henriksen 1987a; Wiesner and Henriksen 1987b). The few studies that have examined the physiological effects of opioids in the dentate gyrus *in vitro* (Haas and Ryall 1988; Tielen et al. 1981) or *in vivo* have focused on field potentials evoked by stimulation of the afferent perforant path. These studies have generally found exogenously applied opioids to exert an "excitatory" effect in that the opioids enhanced evoked potentials, indicating an increased response of the granule cells to input from the perforant path. In this respect, the effect of opioids in the dentate gyrus appears to be similar to the effect of opioids in the hippocampal subfields CA1 and CA3, where evoked potentials are also enhanced (Corrigall 1983). This parallelism of pharmacological effect is reinforced by the finding that, in a fashion similar to that previously discussed for CA1 and CA3, opioids applied to the dentate gyrus decrease sequential inhibition as determined by a "paired-pulse" paradigm (Wiesner et al. 1988). While a disinhibftory action of opioids like that seen in the CA1 and CA3 subfields might be expected in the dentate gyrus, similar effects of opioids on individual dentate granule cells have only been recently reported (Wiesner et al. 1986; Wiesner and Henriksen 1987b; Chavkin et al., this volume). Using a prototypic opioid agonist (D-ala^2-met-5-enkephalin amide (DAMEA)), we have assessed the effects of electrophoretic application on granule cell spontaneous discharge and responsivity to afferent input. Although DAMEA has been used as a standard opioid agonist in other studies, we have determined that selective mu agonists, but not delta or kappa agonists, produce comparable results (Wiesner and Henriksen 1987a) (but see Chavkin et al. (this volume)).

To evaluate recurrent (or feedforward) inhibition characteristics of hippocampal circuitry, a paired-pulse paradigm was employed before and during each opioid application. This paradigm involved pairs of stimulus pulses (a "conditioning" pulse followed by a "test" pulse)

with varying interpulse intervals. Under control conditions, the test response is normally inhibited at short interpulse intervals (less than 50 msec) but facilitated at longer interpulse intervals (50 to 140 msec). During the DAMEA application, the inhibition characteristic of short interpulse intervals was decreased, resulting in significantly greater test responses at 20- and 40-msec interpulse intervals. The decreased inhibition in the presence of opioids resembles that which is induced by certain other opioid peptides (Wiesner and Henriksen 1987a) and is reversed by naloxone. Recordings of single units within the granule cell layer allowed testing of their response to opioid peptides. Electrophoretic application of DAMEA consistently inhibited the spontaneous activity of dentate units while concurrently increasing the amplitude of evoked population spikes (figure 10). When unit responses to perforant path stimulation could be identified superimposed on the field potentials (5 to 15 msec poststimulus), an increased number of evoked action potentials was seen during DAMEA application, suggesting that evoked unit activity was increased even though spontaneous cellular activity was suppressed.

The inhibition of spontaneous unit activity (and the concurrent increase in population spike amplitude) by DAMEA was antagonized by naloxone. The results of this study again confirm that exogenously applied opioids can enhance the excitatory response of the dentate gyrus to perforant path stimulation, and that this effect is associated with a disinhibition of the evoked response as determined by paired-pulse stimulation. In addition, the results have demonstrated a concurrent inhibition of spontaneous granule cell activity by an enkephalin analogue. Although this inhibitory effect of enkephalin is typical of that seen in most areas of the brain (North 1979), it is surprising in light of the opioid effects reported in other hippocampal areas and presents an interesting paradox with respect to the “excitatory” effect of enkephalin on responsiveness to perforant path stimulation. It is possible that these divergent opioid effects may be causally related, although it seems likely that the effects may be mediated through separate mechanisms. While these mechanisms remain obscure, the enhanced response to perforant path input may result from a disinhibitory effect of opioids; this effect could be opposed by a separate, perhaps more direct inhibitory opioid effect. Alternatively, it is conceivable that the decreased spontaneous firing rate may predispose the cell to an increased synchrony of response to incoming stimuli.

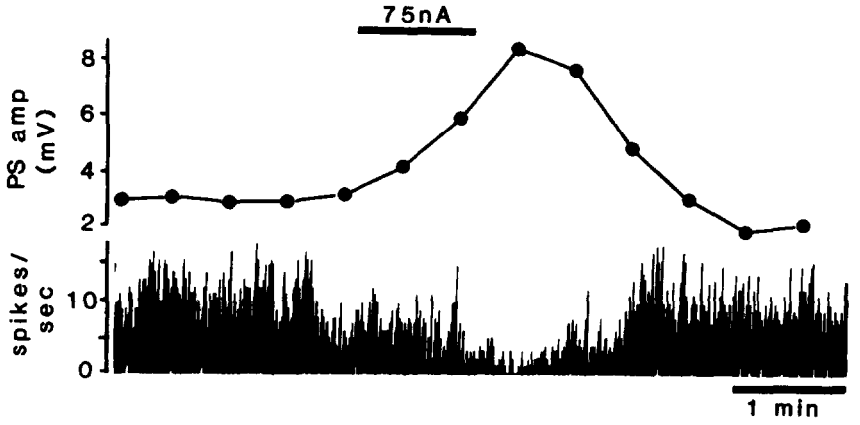

FIGURE 10. *Concurrent effects of DAMEA on population spike amplitude (PS amp) and on spontaneous tiring rate of a single unit of the granule cell layer*

SOURCE: Wiesner and Henriksen 1987a, Copyright 1987, Elsevier Ltd.

These results underscore the importance of caution in interpreting effects of drugs as "excitatory" or "inhibitory" based on studies of evoked potentials alone. Evoked activity such as that reflected by dentate field potentials must be considered a specific measure of transmission within the individual neural pathway involved.

CONCLUSION

It is clear from the foregoing review of *in vivo* opioid neuropharmacology that opiold peptides exist in the hippocampus and can dramatically influence neuronal excitability. The question still remains as to what degree endogenous opioids are functionally involved in hippocampal physiology. However, in light of the data described, the presence of multiple opiate receptors differentially distributed in the hippocampus could explain the complex cellular and EEG effects of the various opioid peptides. These multiple receptors and their distributions could therefore clarify a possible role of opioid peptides in normal hippocampal cellular activity as well as in limbic epilepsy and drug abuse. Hence, opioid peptides of the beta-endorphin-enkephalin class and morphine-like opiate

alkaloids, which act with some degree of selectivity on mu and delta receptors, appear to be predominantly excitatory in the hippocampus. The dynorphins exhibit both excitatory and inhibitory effects, perhaps via actions at both mu and kappa receptors. It seems likely that the predominant receptors involved in opioid effects in the CA1 cell field of the hippocampus and the dentate gyrus are of the mu and delta subtypes, whereas kappa receptors also may be involved in CA3 opioid response. It seems apparent that a multidisciplinary approach will continue to be required before the potential roles of the hippocampal opioids will be finally characterized.

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AUTHORS

Steven J. Henriksen, Ph.D.
James B. Wiesner, Ph.D.

Division of Preclinical Neuroscience
and Endocrinology
Department of Basic and Clinical Research
Research Institute of Scripps Clinic
La Jolla, CA 92037

Guy Chouvet, Ph.D.
Groupe de Recherche de Neurochimie
Functionnelle
INSERM
St. Genis-Laval, France

Opioid Receptor Mechanisms in the Rat Hippocampus

Charles Chavkin, John F. Neumaier, and Elizabeth Swearengen

INTRODUCTION

When the actions of opiates were measured on individual neurons, the predominant effects seen throughout the nervous system were membrane hyperpolarization and decrease in firing rate (Nicoll et al. 1977; Duggan and North 1984; Henderson 1983). An opioid-induced increase in excitability shown by hippocampal CA1 pyramidal cells was therefore viewed as an anomaly. The ionic basis for opioid actions outside the hippocampus has been shown to be an increase in membrane conductance to potassium following activation of mu or delta opioid receptors, or alternatively, a decrease in a voltage-dependent calcium conductance following activation of kappa opioid receptors. Investigation of the response to opioids within the CA1 region of the hippocampus revealed that the direct action of opioids was also an increase in potassium conductance (North 1986). To account for opioid-Induced excitation in the hippocampus, several groups have proposed that opioids hyperpolarize a population of interneurons and thereby inhibit the release of an inhibitory transmitter that normally controls the excitability of the CA1 pyramidal cells (Henderson 1983). In this article, we review some Of the recent evidence supporting this hypothesis and present data suggesting that opioids act via a similar cellular mechanism in the dentate and CA3 regions of the hippocampus.

IONIC BASIS OF OPIOID ACTION

Wen and Macdonald characterized the opioid receptors and ionic transduction mechanisms involved in mediating the actions of

opioids by studying the electrophysiological effects of opioids applied to fetal mouse dorsal root ganglion (DRG) cells in culture (Wen and Macdonald 1983; Wen and Macdonald 1984; Macdonald and Wen 1986). In this preparation, opioids decrease calcium-dependent action potential duration (Werz and Macdonald 1983), as was reported previously for cultured chick DRG cells (Mudge et al. 1979). In addition, kappa-specific opioids also selectively reduced the voltage-sensitive calcium conductance (Werz and Macdonald 1984; Werz and Macdonald 1985); however, the mechanism of action for kappa receptors was different from that for mu or delta receptors. Intracellular recordings showed that electrodes filled with cesium acetate (blocking potassium conductances) completely inhibited the effects of [Leu^5]enkephalin (delta agonist) and morphiceptin (mu agonist), whereas cesium did not affect the reduction in calcium conductance caused by dynorphin-A(1-17), dynorphin-A(1-8), dynorphins, and alpha-neoendorphin (kappa agonists). The authors therefore concluded that kappa receptors primarily control a calcium channel in mouse DRG cells, whereas delta or mu receptor activation decreases calcium conductance by directly increasing potassium conductance (Werz and Macdonald 1984; Wen and Macdonald 1985).

Opioid receptor-linked conductance changes were also measured in guinea pig myenteric neurons. Neurons in the myenteric ganglia respond to application of mu-selective opioids with a 3-to 7-mV hyperpolarization accompanied by a decrease in membrane resistance (Morita and North 1982). Opioids also prolong the calcium-dependent hyperpolarization that normally follows a burst of action potentials ($I_{K(Ca)}$) (Tokimasa et al. 1981). These effects were shown to be mediated by mu receptor activation by the sensitivity of a variety of opioids to antagonism by beta-funaltrexamine (beta-FNA) (Cherubini and North 1985), a mu-receptor-alkylating agent (Portoghese et al. 1980; Takemori et al. 1981). Several observations demonstrated that the ionic basis of the hyperpolarization is an opioid-induced increase in potassium conductance: (1) the amplitude of the hyperpolarization depends on the external potassium concentration: (2) the reversal potential of the opioid-activated current is about -100 mV, a value close to the potassium equilibrium potential of these cells (Morita and North 1982; Tokimasa et al. 1981); and (3) potassium channel blockade by intracellular cesium injection blocks the mu receptor-mediated hyperpolarization (Cherubini and North 1985).

Kappa agonists (e.g., dynorphin-A(1-17), tifluadom, and U50,488H) affect guinea pig myenteric neurons differently than delta and mu agonists. Rather than membrane hyperpolarization, cells respond with a reduction in the duration of the calcium-dependent action potential (Cherubini and North 1985). Kappa agonist action is distinguishable from mu receptor activation in the same preparation by the insensitivity of kappa agonists to beta-FNA treatment, and by either the intracellular injection of cesium or the addition of low concentrations of barium to the super-fusion buffer to block potassium channels (Cherubini and North 1985; Cherubini et al. 1985). The results of experiments using these techniques suggest that kappa receptors are present on myenteric neurons and are coupled to calcium channels. Thus, kappa and mu receptors in this tissue, as in mouse DRG neurons, are apparently linked to different transduction mechanisms, although both may reduce calcium influx during the action potential.

Analysis of opioid effects on locus coeruleus neurons in rat brain slices demonstrates that mu receptor activation causes a membrane hyperpolariration of these neurons as well (Pepper and Henderson 1980; Williams et al. 1982; Williams et al. 1984). Experimental manipulations similar to those done for the guinea pig myenteric neurons show that the membrane hyperpolarization is due to an increase in potassium conductance. In addition, quinine (200 μM), which blocks specific potassium channels in vertebrate and invertebrate neurons (Walden and Speckmann 1981; Yoshida et al. 1986), also blocks the opioid-activated potassium conductance in locus coeruleus neurons (North and Williams 1985). These data indicate that mu opioid receptors are linked to similar transduction mechanisms not only in different tissues but in different species as well.

Delta receptors have been characterized in neurons of the submucous plexus of the guinea pig ileum. These neurons were hyperpolarized following application of delta agonists, [Met^5]enkephalin, [Leu^5]enkephalin, [D-Ser^2,L-Leu^5,L-Thr^6]enkephalin (DSLET), or [D-Ala^2,D-Leu^5]enkephalin (DADLE) (Surprenant and North 1985; Mihara and North 1986). Opioid effects on the submucous plexus neurons were found to be mediated by delta receptors as shown by the apparent dissociation constants of naloxone and ICI-I 74864 calculated by Schild analysis (Schild 1947; Schild 1949) and by the insensitivity of the cells to either mu or kappa agonists. The conductance change was very similar to that controlled by mu receptor

activation in the myenteric plexus (i.e., hyperpolarization that reversed at the potassium equilibrium potential and appeared to result from an outward potassium current).

Based on studies of the submucous plexus *in vitro* and of DRG cells in culture, the properties of the delta-receptor-activated potassium channel were indistinguishable from the mu receptor channel. The similarity In mechanism may reflect a close evolutionary relationship between the mu and delta receptors and suggests that there may be other common features. In contrast, kappa receptors have been shown to control calcium conductance. However, the net effect *on* cell excitability may be the *same* regardless of which mechanism is activated. Increased potassium conductance reduces excitability of the postsynaptic cell and decreases transmitter release at the nerve terminal. Inhibition of calcium conductance also reduces postsynaptic cell excitability by reducing the calcium component of the action potential. Reduction of transmitter release may result if kappa receptors are also located on the nerve terminals.

ENDOGENOUS OPIOID IN THE HIPPOCAMPUS

Interest in the actions of opioids in the CA3 region of the hippocampus was greatly stimulated by the immunocytochemical localization of proenkephalin- and prodynorphinderived opioids in the dentate gyrus and mossy fiber pathway of the rat hippocampus (Gall et al. 1981; Gall 1984; McGinty et al. 1983; McGinty et al. 1984). Neurochemical and immunohistochemical studies of the hippocampus, dentate gyrus, and entorhinal cortex suggest the presence of two separate opiold peptide projection systems in this complex (Chavkin et al. 1985k Gall, this volume: McGinty, this volume).

To identify the molecular forms and quantify the regional contents of the dynorphin-A and [Leu^5]-enkephalin immunoreactivity, peptide extracts of microdissected hippocampal regions were resolved by reversed phase high-pressure liquid chromatography (HPLC) and molecular exclusion HPLC (Chavkin et al. 1983b). These studies demonstrated that the dynorphin-A and [Leu^5]enkephalin immunostaining corresponded to authentic dynorphin-A(1-17) and [Leu^5]enkephalin. The probable neurotransmitter roles of [Leu^5]enkephalin and dynorphin-A were further supported by our demonstration of calcium-dependent release of these peptides after in vitro depolarization of hippocampal tissue (Chavkin et al. 1983a).

Release of putative transmitters from hippocampal slices was induced by cellular depolarization using 50 mM KCl, veratridine, or kainic acid added to the super-fusion buffer. The effluents were assayed using specific radioimmunoassays for [Leu^5]enkephalin and each of the five known prodynorphin derived opioids. Although transmitter release induced by chemical depolarization may not precisely mimic endogenous release stimuli, the results of these experiments show that each of the endogenous dynorphin oploids is present in the hippocampus and has the potential of being released by physiological stimuli.

Thus, certain minimal criteria necessary to establish a neurotransmitter role of the oploid peptides in the hippocampus have been satisfied. Anatomical methods have identified the distribution of the peptides within the hippocampus, and depolarization-induced release of opioids has been shown. Opioid binding sites are present in this region as demonstrated by membrane binding and slice autoradiography (Herkenham and McLean, this volume). Further characterization of the neurotransmitter role of the opioids in the hippocampus requires that the actions of opioids be more completely understood, that the cellular distribution and types of the receptors mediating those effects be defined, and that endogenous opioid release be physiologically demonstrated. Significant progress has been made toward these goals.

OPIOID ACTIONS IN THE CA1 REGION

Numerous groups have studied the electrophysiological effects of opioids in the CA1 region of the rat and guinea pig hippocampi (see Henderson (1983), Duggan and North (1984) for review). Most opioids increase pyramidal cell firing rates and increase the electrical excitability of the CA1 region. The latter effect is most clearly demonstrated by electrical stimulation of afferent fibers (Schaffer collaterals) in the stratum radiatum. The effect of opioids under these conditions is to consistently increase the sensitivity of these cells to electrical stimulation measured by a decrease in the stimulation intensity required to evoke a response. Another consistent opioid effect is the generation of secondary responses which follow the primary population spike (figure 1). Despite these easily demonstrated effects on excitability, intracellular recordings have shown that opioids do not change intrinsic membrane properties of CA1 pyramidal cells (Dingledine 1981; Siggins and Zieglgänsberger 1981).

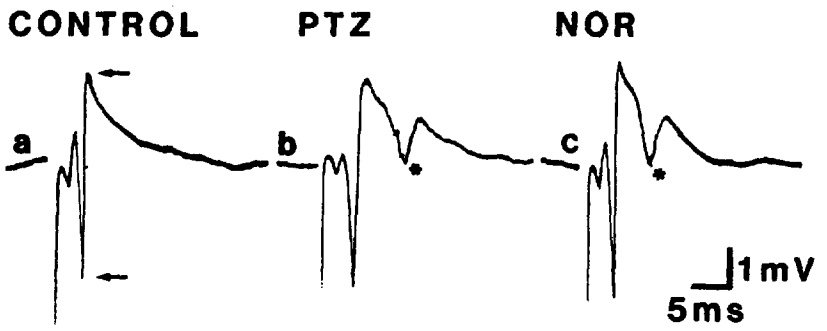

FIGURE 1. *CA1 pyramidal cell population responses evoked by afferent stimulation of Schaffer collaterals*

NOTE: The stimuiating electrode was placed in the center of the stratum radiatum, and the recording electrode was placed at least 1 mm away in the CA1 pyramidal cell layer. The figure is marked as follows: (a) control, (b) 2 mM pentylenetetrazole, and (c) 1 μM normorphine. Arrows mark the primary population spike, and asterisks mark the afterpotential. Drugs were added to the superfusion buffer bathing the tissue and allowed to reach steady state concentration before making experimental measures (10 minutes). Drug effects were completely reversible within 10 to 60 minutes. The size and number of afterpotentials are dose dependent.

The mechanism of the opioid effect on CA1 pyramidal ceil excitability has been studied by several groups and is thought to be largely due to reduction of inhibitory input (e.g., excitation resulting from disinhibition) as proposed by Zieglgänsberger et al. (1979). Extracellular recording of CA1 interneurons *in vitro* support this hypothesis by showing that opioids directly reduce firing rates and excitability (Lee et al. 1980). As predicted by the disinhibition hypothesis, DADLE and other opioids applied to the hippocampal slice were found to directly hyperpolarize intemeurons by increasing their potassium conductance, consistent with the effects of delta receptor activation in myenteric neurons (Nicoll and Madison 1984). The resulting reduction in excitability and firing rate of the intemeurons could decrease gamma-aminobutyric acid (GABA) release and reduce inhibitory postsynaptic potential (IPSP) amplitude recorded in pyramidal ceils.

Raggenbass et al. (1985) also studied the effects of opioids on nonpyramidal ceils in the CA1 region of the rat hippocampus. [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (DAGO) (a mu-selective agonist) but not U50,488H (a kappa-selective agonist) suppressed the firing of these cells. This action was direct, since elevated magnesium and reduced calcium in the superfusion medium did not Mock the effects of DAGO. The authors concluded that the mu receptor type was activated, as the effect was shown to be produced by DAGO but not U50,488H and was Mocked by naloxone. Presumably, the inhibitory transmitter affected by opioids is GABA, aithough a direct neurochemical measure of the effect of [D-Ala^2]enkephalin-amide on endogenous GABA release from hippocampal slices failed to find any reduction in stimulated release (Fan et al. 1982).

To further test the disinhibition hypothesis of opioid action, it is necessary to investigate the actions of opioid agonists in relation to GABA and excitatory amino acid receptor antagonists. if opioids increase CA1 pyramidal excitability by directly inhibiting GABA release from interneurons, then GABA antagonists should mimic opioid effects. This is the case; a variety of GABA receptor antagonists such as pentylenetetrazole, bicuculine, and picrotoxin produce multiple population bursts in response to afferent stimulation (Herron et al. 1988; Hablitz and Langmoen 1986; Ashwood and Wheal 1986). GABA antagonists reduce the IPSP and increase the excitatory postsynaptic potential (EPSP), resulting in sufficient depolarization to unmask N-methyl-D-aspartate (NMDA) receptors (Herron et al. 1986; Hynes and Dingledine 1984; King and Dingiedine 1985). NMOA receptors are a specific subset of excitatory amino acid receptors named for their most potent agonist (Watkins and Evans 1981). During moderate stimulation of synaptic afferents, NMDA receptors are not activated in the hippocampus. D-2-amino.-5-phosphono-valerate (D-APV), a selective NMDA receptor antagonist, has no effect by itself on synaptic responses (Koerner and Cotman 1982; Collingridge et al. 1983; Ashwood and Wheal 1986). However, several NMDA receptor antagonists display potent anticonvulsant activity in a variety of *in vivo* models of epilepsy (Croucher et al. 1982; Meldrum et al. 1983), and D-APV was shown to completely block CA1 pyramidal cell afterpotentials produced by pentylene-tetrazole (Herron et al. 1988).

We compared CA1 pyramidal cell population responses evoked in the absence and presence of the GABA receptor antagonist pentylene-tetrazole with responses evoked by the mu-selective opioid receptor

agonist normorphine (figure 1) (Swearengen and Chavkin 1987). In both cases, drug application resulted in the appearance of a second population burst (afterpotential) following the primary population spike. As predicted, D-APV partially blocked normorphine-induced afterpotentials in CA1 pyramidal cell population responses (figure 2), but did not affect the opioid-induced increase in sensitivity of

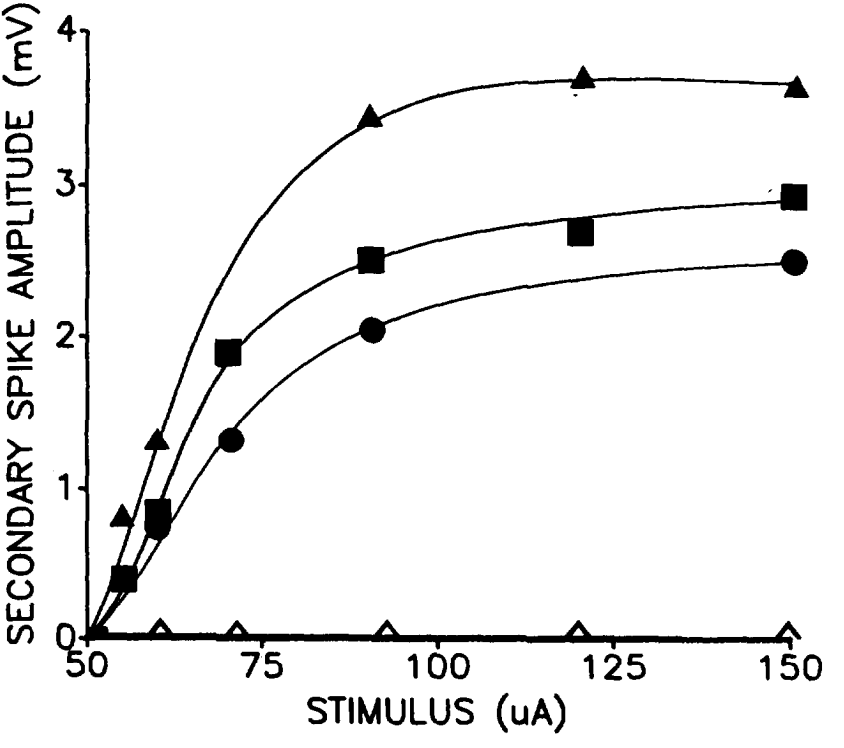

FIGURE 2. *Effect of D-APV on stimulus-response characteristics of normorphine-induced afterpotentials*

NOTE: The stimulation and recording protocol was identical to that described in figure 1. Application of normorphine (10 µM) to the tissue elicited a single afterpotential as in figure lc. in the absence of normorphine, the afterpotential was completely absent (open Mangles). The maximum afterpotential amplitude was dependent on the concentration of normorphine in the superfusion buffer (closed triangles). Addition of D-APV to the superfuslon buffer partially blocked the stimulation-dependent rise in afterpotential amplitude (squares, 16 µM D-APV circles, 50 µM D-APV

the primary population spike to afferent stimulation (data not shown). Thus, the mechanism of opioid action must include, but not be limited to, an unmasking of NMDA receptors on the CA1 pyramidal cells.

To further define the types of receptors that mediate opioid action in the CA1 region, we used a selective receptor inactivation paradigm (Chavkin et al. 1985a). In that study, we measured the ability of opioids to increase CA1 pyramidal cell sensitivity to electrical stimulation after pretreatment of the rat hippocampai slice *in vitro* with the irreversible mu-selective antagonist beta-FNA. Beta-FNA-treated slices were insensitive to the effects of normorphine and [N-MePhe^3,D-Pro^4] morphiceptin (PL017). Moreover, the effects of both dynorphin-A and dynorphin-B were almost completely blocked, suggesting that these agonists activate mu receptors in CA1. In contrast, the responses to DADLE and DSLET were not significantly changed by beta-FNA treatment. Our interpretation of these results is that both mu and delta opioid receptors are involved in the specific response to opioids in the CA1 region.

Additional receptor characterization was achieved by Schiid analysis (Neumaier and Chavkin 1988; Neumaier et al. 1988). As expected for compounds acting through mu opioid receptors, normorphine, PLO17, and dynorphin-A(1-17) were found to be highly sensitive to naloxone antagonism in CA1 (apparent dissociation constants were between 1 and 3 nM for each) (figure 3) (Neumaier and Chavkin 1986; Neumaier et al. 1986). in contrast, the naloxone sensitivity of DSLET was significantly lower than that of PLO17 (4.5 nM, $P<0.05$, $n=8$).

These results support the conclusion that opioids in the CA1 region of the rat hippocampus activate mu and delta but not kappa receptors, and that at moderate concentrations the dynorphin opioids can activate mu receptors (Chavkin et al. 1985a). Although the population of opioid receptors acted on by DSLET is different from that acted on by normorphine, PLO17, and dynorphin-A, the extracellularly measured response to DSLET is very similar to that found with mu-selective agonists (figure 4a, b, and c).

In analyzing opioid effects in the CA1 region, it is important to address the disagreement among the published reports on the effects of dynorphin-A(1-17). We consistently see excitatory actions of dynorphin-A in the rat hippocampal slice preparation

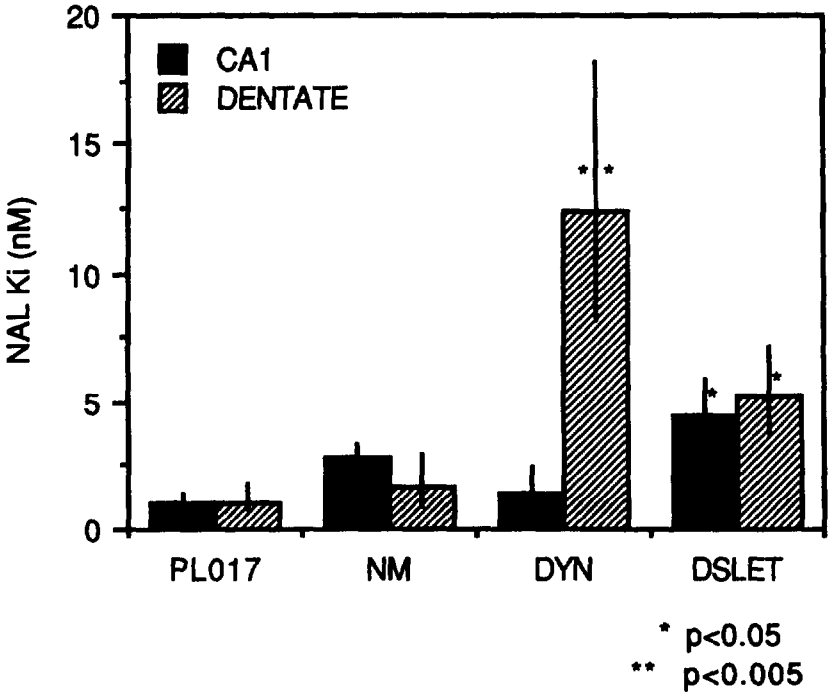

FIGURE 3. *A comparison of the apparent dissociation constants of naloxone at receptors activated by opioid agonists in the CA1 and dentate regions*

NOTE: Schild analysis of naloxone-induced shifts of agonist dose-response curves yielded apparent dissociation constants (K_i values) for naloxone at the receptor activated by each agonist used. Each value is the geometric mean plus or minus SEM of two to eight experiments. The mu-selective agonists normorphine and PL017 were used to determine the naioxone K_i at mu receptors; these values were not significantly different from one another (ranked sum test). The K_i associated with DSLET was significantly greater than that for PL017 ($p<0.05$) in both CA1 and dentate regions. The dynorphin-A(1-17)-related value in CA1 was not significantly different from that for the mu agonists; however, in the dentate gyrus, the dynorphin-related value was significantly larger than that for PL017 in dentate ($p<0.01$) and dynorphin in CA1 ($p<0.05$).

(Chavkin et al. 1985a; Neumaier and Chavkin 1986; Neumaier et al. 1986). Others report that dynorphin-A(1-17) is excitatory at low

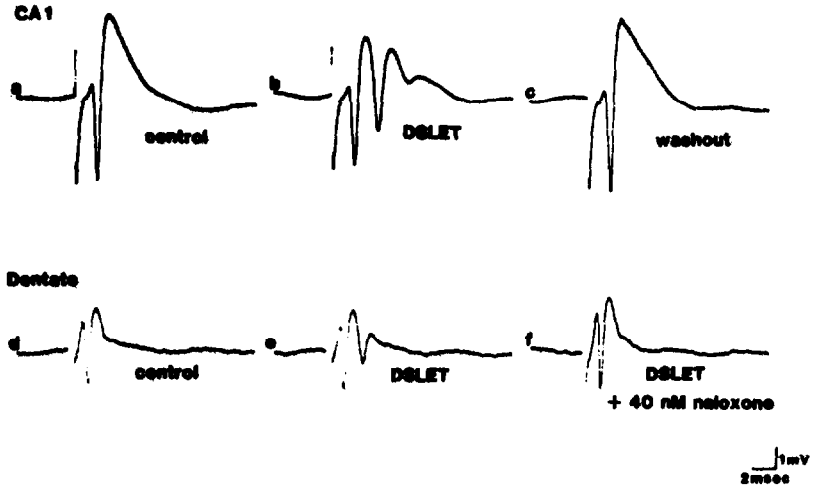

FIGURE 4. *DSLET causes afterpotentials in CA1 and dentate regions*

NOTE: Extracellular recordings were made from CA1 pyramidal cells (a, b, and c) and dentate granule cells (d, e, and f) as described in the preceding text, in both regions, control responses consisted of a stimulus artifact followed by a fiber potential and a single synaptic population spike (a, d). DSLET was applied using micropressure pipetes placed very close to the recording site. The addition of DSLET (1 second, 40 psi, 100 µM) caused a secondary afterpotential in a dose-dependent manner in both region (b, e). The DSLET effect reversed completely after a washout of 1 to 3 minutes (c,, data not shown for dentate) and was completely blocked by naloxone (40 nM) added to the superfusion bath (f, data not shown for CA1).

concentrations (0.1 nM) but inhibitory at higher concentrations (Vidal et al. 1984). It is crucial to note that, following *in vivo* iontophoretic application of dynorphin-A(1-17), inhibition of extracellularly recorded single-unit firing rate was not reversed by naloxone (Walker et al. 1982; Henriksen et al. 1982; Moises and Walker 1995). Des-tyr-dynorphin-A(2-17), *a* dynorphin analogue lacking opioid receptor affinity (Chavkin and Goldstein 1981), had similar inhibitory effects that were insensitive to naloxone (Walker et al. 1982). Thus, low to moderate concentrations (0.1 nM to 1.0 µM) of dynorphin-A(1-17) can activate mu receptors, producing typical excitatory effects, while at higher concentrations pyramidal

cell activity is depressed by a nonspecific and naloxone-insensitive mechanism.

In summary, current evidence indicates that the excitatory effects of opioids in the CA1 pyramidal cell region are due to the presence of functional mu and delta opioid receptors on nonpyramidal cells. The question of whether single interneurons express both mu and delta receptors has not yet been resolved. The data suggest that CA1 intemeurons respond to opioids by increasing potassium conductance; the resulting, hyperpolarization may reduce GABA release in the region. This reduction in inhibition of the pyramidal cell leads to activation of NMDA receptors which mediates at least one component of pyramidal cell excitation.

OPOID ACTIONS IN THE CA3 REGION

In the CA3 region, [Leu^5]enkephalin and DADLE consistently increase pyramidal cell excitability both *in vivo* (Henriksen et al. 1982; Moises and Walker 1985) and *in vitro* (Masukawa and Prince 1982; Gruol et al. 1963). In contrast, dynorphin-A(1-17) has variable effects on CA3 pyramidal cell excitability (Gruol et al. 1963; Chavkin and Bloom 1986; lwama et al. 1986).

Henrisen et al. (1962) studied the effects of iontophoretic and micropneumatic application of the dynorphins on hippocampal pyramidal cell firing rates *in vivo.* The excitatory dynorphin effects appear to be mediated by an opioid receptor, because the' nonopioid analogue des-tyr-dynorphin-A(2-17) was inactive, and the excitatory effects were blocked by systemically or iontophoretically applied naloxone. In comparison, Bradley and Brookes reported that the inhibitory responses to iontophoretically applied dynorphin-A(1-13) *in vivo* were blocked by naloxone (Bradley and Brookes 1984; Brookes and Bradley 1984). Interpretation of this study is difficult, however, because the exact region of the hippocampus (e.g., CA1 , CA3, or dentate) was not identified. In addition, these findings are not consistent with those of Walker et al. (1982) who reported that, in the CA3 region, dynorphin-A(1-17) had only naloxone-insensitive inhibitory effects. In their hands, des-tyr-dynorphin also reduced the CA3 spontaneous firing rate, but the observed inhibition was not naloxone reversible.

The results obtained *in vitro* have also been controversial. We have compared the effects of dynorphin-A(1-17) and [Leu^5]enkephalin on CA3 pyramidal cell excitability using extracellular field potential recording methods in the hippocampal slice (Gruol et al. 1963). [Leu^5]enkephalin consistently enhanced the pyramidal ceil responses. However, in different slices, dynorphin-A(1-17) either increased or decreased pyramidal sensitivity to mossy fiber stimulation. One example of a clear excitatory effect is shown in figure 5: dynorphin-A(1-17)-induced afterpotentials were completely blocked by 20 nM naloxone. The basis for this inconsistency is not known, but it may be due to differences in viability among the slices.

Iwama et al. (1966) similarly reported mixed effects of dynorphin-A(1-17) on guinea pig hippocampal slices. In that study, the depressions produced by dynorphin-A(-17) were also produced by very high concentrations (100 μM) of U50,488H and bremazocine and were not reversed by naloxone (100 μM). The authors suggest that the inhibitory action may be mediated by kappa receptors. However, the low potency of kappa agonists and the insensitivity of the effect to naloxone antagonism indicate that the observed depression was not likely to have been mediated by an opioid receptor.

To further investigate the actions of dynorphin-A(1-17) in the CA3 region, we have recorded intracellularly from hippocampal pyramidal cells *in vitro* (Gruol et al. 1963). Dynorphin-A(1-17) added to the superfusion buffer produced variable effects on the spontaneous activity, burst discharges, and membrane potential. Neither orthodromically evoked synaptic potentials nor resting input resistance were significantly altered by dynorphin-A(1-17). In this system, [Leu^5]enkephalin reduced IPSPs with no change in membrane potential or input resistance, in accord with previous studies in CA1 (Masukawa and Prince 1982). The predominant effect we observed using dynorphin-B(1-13) was a moderately long duration (0.1 to 10.0 seconds) depolarizing response of 5 to 20 mV that was completely blocked by naloxone (Chavkin and Schwartzkroin, unpublished observations). No consistent effect of dynorphin-B was seen on the other components of the spontaneous or synaptically activated responses including IPSPs evoked by mossy fiber stimulation or the after hyperpolarization following intrinsic bursts.

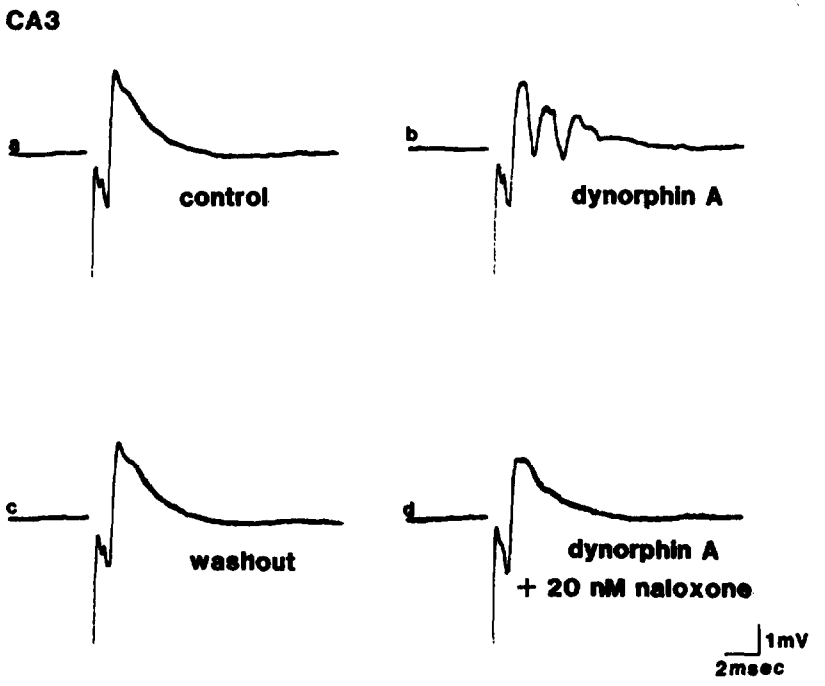

FIGURE 5. *Dynorphin-A(1-17) causes after potentials in CA3*

NOTE: Guinea pig CA3 pyramidal cells were electrically stimulated In the mossy fiber tract (150 µA), and their activity was recorded extracellularly in the stratum pyramidale. Control responses showed no afterpotentials (a). Following application of dynorphln-A(1-17) (1 second, 40 psi, 100 (µM), multiple afterpotentials were generated (b). These afterpotentials disappeared completely following 1 to 3 minutes of washout (c) and were completely blocked by 20 nM naloxone (d). Schild analysis of the shift in dynorphln-A(1-17) dose-response curves with bath superfusion of naloxone at 10, 20, and 40 nM yielded an apparent dissociation constant of 4.8 nM (data not shown).

The basis for the different findings reported by different laboratories is unclear but may be due to differences in tissue preparation or method of peptide application. It is also possible that the potent nonspecific actions of the dynorphin peptides may obscure

the specific, opioid-receptor-mediated actions under the experimental conditions used. We expect that the dynorphins have presynaptic disinhibitory effects in CA3 based on the typical inhibitory actions of opioids seen in other brain regions. However, neither the receptor types present nor the ionic basis for the opioid actions in the CA3 region have yet been established.

OPIOID ACTIONS IN THE DENTATE REGION

Since dynorphin and enkephalin peptides are present in the dentate granule cells and their processes, it is possible that these peptides are released from dentate granule cell collaterals to modulate neuron excitability within the dentate gyrus. In addition, the proenkephalin-derived opioids present in the perforant pathway are likely to be released at terminals in the dentate molecular layer. Intracellular and extracellular recordings as well as behavioral data indicate that opioids have effects on the dentate granule cells (Haas and Ryall 1980; Tielen et al. 1981; Linseman and Corrigall 1982; Collier and Routtenberg 1984). Using rat and guinea pi hippocampal slices, Tielen et al. (1961) found that [D-Ala^2,Met^5]enkephalin added to the superfusion buffer caused a naloxone-sensitive depression of the evoked response at peptide concentrations between 0.3 and 1.0 µMI.

We find that extracellular recording in the granule cell layer following orthodromic perforant path stimulation reveals a stimulus-response relationship that differs from that found in CA1 or CA3 (figure 6). Whereas the stimulus-response cures always plateau in CA1 and CA3, a biphasic relationship is often observed in the dentate region. The biphasic pattern may reflect the recruitment of inhibitory input to granule cells at high stimulus intensities. Both the inhibition and the excitation have been shown to be sensitive to opioids (Tielen et al. 1981; Neumaier et al. 1986). Our findings suggest that the main effect of opioids on the primary population spike in the dentate gyrus was to transform the stimulus-response relationship from a biphasic to a sigmoidal shape without an apparent increase in sensitivity to electrical stimulation of the primary spike (figure 6). This is different from the opioid-induced shift to the left of the stimulus-response curves seen in CA1 and CA3 and may reflect differences in the synaptic circuitry.

An additional naloxone-sensitive effect of opioids in the dentate gyrus was the dose-dependent induction of afterpotentials (Neumaier

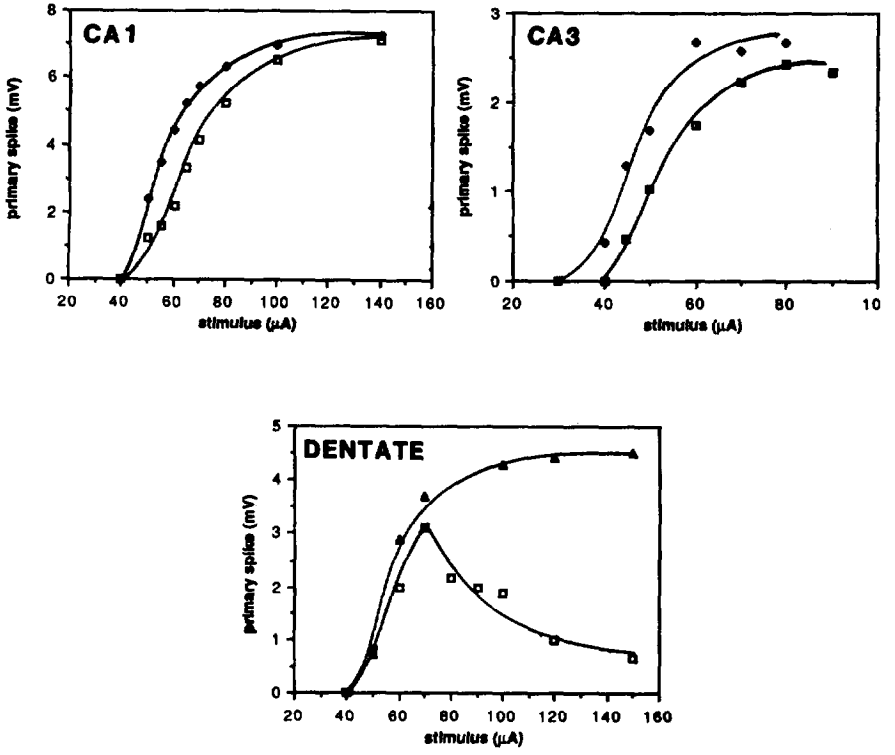

FIGURE 6. *Stimulus-response curves in the hippocampus are altered by opioids*

NOTE: Stimulus-response curves were generated by measuring the amplitude of the primary spike while varying the intensity of electrical stimulation. In each region, a control curve was constructed, an opioid was introduced, and then a new stimulus-response curve was measured. In CA1 and CA3, normorphine (5 μM) added by bath superfusion caused a shift to the left in the stimulus-response relationship with no change in the maximum response. In the dentate gyrus, dynorphin-A(1-17) added by pressure micropipette (1 second, 40 psi, 100 μM) did not shift the curve to the left, but changed the curve from a biphasic to a sigmoidal shape.

and Chavkin 1988; Neumaier et al. 1988). The effect was reversed 1 to 5 minutes after drug application by micropipette; it was also completely blocked by the addition of naloxone (10 to 100 nM) to the superfusion buffer (figure 4). We quantified opioid action by measuring the amplitude of the afterpotential recorded in the

granule cell layer. Schild analysis of normorphine sensitivity to naloxone antagonism yielded an apparent dissociation constant of 1.6 nM (0.9 to 2.6, n=4). This value is very close to the apparent affinity of naloxone for the mu opioid receptor (Lord et al. 1977). Similarly, the apparent dissociation constant of naloxone derived from the shift in PL017 (a mu-selective agonist) was 1.0 nM (0.6 to 1.7, n=4). These values agree well with the results from similar experiments using the same drugs in CA1 (figure 3) and suggest that normorphine and PL017 act through the mu receptors in the dentate region of the hippocampus.

Using the extracellular recording paradigm described above, we measured the effects of dynorphin-A(1-17) following application by micropipette. Dynorphin-A(1-17) also caused the appearance of an afterpotential in the dentate granule cell layer response (Neumaier and Chavkin 1986; Neumaier et al. 1988). The dynorphin effect was reversed by naloxone but required a higher concentration than that required to antagonize completely the actions of normorphine or PL017. The apparent dissociation constant of naloxone in these experiments was 12.3 nM (8.4 to 18.1, n=8), which was significantly larger than that associated with PL017 ($p<0.01$, ranked sum test). The resistance of dynorphin-A(1-17) effects to naloxone in the dentate region suggests that these effects are mediated by kappa receptors (Neumaier and Chavkin 1986; Neumaier et al. 1986). In similar experiments (figure 4d, e, and f), we found that DSLET-induced afterpotentials were inhibited by naloxone with an apparent dissociation constant of 5.2 nM (n=3). Although this value is lower than that typically seen with delta receptor activation (Lord et al. 1977), the naloxone sensitivity of DSLET was significantly higher than that found for PL017 in the same assay. The receptor types activated by DSLET are not yet known. We can speculate that the intermediate naloxone sensitivity indicates that DSLET activates both mu and delta receptors under these assay conditions.

CONCLUSIONS

An image of endogenous opioid action in the hippocampus is emerging. Disinhibition of the principal cell type is the most consistent effect of pharmacologically applied opioids in each of the three major divisions of the hippocampal formation. This was most clearly demonstrated in the CA1 region, and recent studies have documented similar actions in CA3 and the dentate gyrus. Additional experimental details are necessary to define precisely the

cell type expressing the opioid receptors, the location of the receptors on the cell, and the transmitter(s) whose release is controlled by opioid receptor activation. while similarity among the regions is evident, differences have also been noted in the responses to opioid application; thus, differences in the synaptic circuitry are likely.

The analysis of opioid action in the hippocampus continues to require an understanding of the conditions under which endogenous opioids are released and the cellular targets of the released opioid. In addition, we will need to compare the actions of endogenously released enkephalins and dynorphins with the effects of pharmacological activation of opioid receptors. Ultimately, this information will provide a clearer view of the role of endogenous opioids in controlling the excitability of hippocampal neurons and information transfer through this brain region.

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AUTHORS

Charles Chavkin
John F. Neumaier
Elizabeth Swearengen

Department of Pharmacology
University of Washington
Seattle, WA 98195

Behavioral Significance of Opioid Peptides in Relation to Hippocampal Function

Michela Gallagher

INTRODUCTION

The first reference to an action of opiates on learning and memory can be found in a report published in 1920 (Macht and Mora 1920). This archival study would seem a fitting background for considering some behavioral implications of the hippocampal opioid peptides described in previous chapters. These early investigators reported that low (subanalgesic) doses of morphine impaired a "memory habit" in rats tested on a circular maze. This observation echoes two prominent themes in contemporary behavioral studies on hippocampal function. On the one hand, the results from a variety of research studies point to the role of the hippocampus in memory (Mishkin 1982; Olton 1983; Zola-Morgan et al. 1988). At the same time, much evidence indicates that rat hippocampal neurons process spatial information such as that required in many maze-learning tasks (O'Keefe and Nadel 1978).

This inquiry will begin by considering the effects of altering opioid peptide function on learning and memory processes that are associated with the hippocampus. A second line of inquiry will focus on the possibility of oploid peptide regulation of afferents to the hippocampal formation. Primarily, my remarks will address evidence that septohippocampal choiinergic (acetylcholine (ACh)) function is under opiold peptide control. Finally, some preliminary evidence will be discussed indicating that training procedures, which engage hippocampal function, might be used to monitor opioid peptide activity in this structure.

OPIOID PEPTIDE REGULATION OF MEMORY FOR SPATIAL INFORMATION

Ample evidence is now available indicating that opiate treatments alter learning and memory across a wide array of tasks and species (see recent review by Gallagher (1985)). With one notable exception discussed later in this chapter, the results from this work are quite uniform. in general, low doses of opiate agonists and pep tides have detrimental effects, whereas opiate antagonist administration, by itself, augments the effects of training on acquisition and retention of new learning. Drug treatments can produce these effects when administered either prior to training or during an interval after the training experience is completed. When examined, the effects of opiate treatments are found to exhibit stereospecificity and to be shared by a series of compounds with either agonist or antagonist properties.

Quite a few studies in this line of work have used testing procedures that are also sensitive to treatments which affect the hippocampal formation. For example, several studies have examined the effects of opiate antagonists on memory-based performance using spatial tasks that are sensitive to hippocampal damage. In one such study, Collier and Routtenberg (1984) tested rats on a radial arm maze task in which animals were trained to remember the location of a food reward over a delay period. Either drug or vehicle injections were given prior to sessions when presentation of a food reward at one location on the maze was followed 20 minutes later by a retention test, when animals were again rewarded if they returned to the same location on the maze. The rewarded location varied among the eight arms of the maze across test sessions. Administration of naloxone was found to facilitate accurate performance: following drug treatment, animals committed fewer errors after the delay in returning to the previously rewarded location.

Rats with hippocampal damage fail to perform accurately on this maze task (Kesner and DiMattia 1984). Interference with normal hippocampal function produced by intracranial electrical stimulation likewise impairs performance (Bierley et al. 1983; Collier and Routtenberg 1984). Indeed, in their study, Collier and Routtenberg (1984) found that electrical stimulation of the dentate gyrus after the training trial impaired subsequent retention. In addition, this effect of hippocampal stimulation was prevented by prior systemic administration of naloxone. At the time, these investigators

speculated that the disruptive effects of dentate stimulation on memory might be due to the release of opioid peptides from dentate granule cells.

In our laboratory, we have developed a somewhat different version of the radial arm maze task to examine the effects of opiate treatments on memory-based performance using spatial information. Animals are trained to visit each arm of the maze once in a session in one spatial environment. The experiment is then conducted by examining acquisition of criterion performance on the maze in a series of new spatial environments. In each new environment, probe trials are conducted to confirm that the accurate performance which develops over several training trials depends on spatial cues specifically located around the maze in the new room. In each new spatial environment, a treatment is given-either drug or vehicle. Injections are administered after removing the animal from the maze 5 or 8 hours prior to testing subsequent performance. We have found highly reliable and robust improvement in acquisition of criterion performance on this task when opiate antagonists are given. The data in table 1 are collected from several experiments and show this effect with systemic administration of several different opiate antagonist agents (Fanelli et al. 1985; Gallagher et al. 1983; Gallagher et al. 1985a).

In these studies using rats, opiate antagonists do not disrupt functions that presumably depend, at least in part, on hippocampal circuitry. Indeed, there is no evidence from this work that activation of these or other opioid peptides supports or improves the behaviors monitored in these experiments; when opiate antagonists are given, the results uniformly reveal improved performance. Based on these findings, we might even speculate that activation of hippocampal opioid peptides might restrain or diminish the learning/memory capacities that depend on hippocampal processing; but, of course, none of these experiments have shown that antagonists act by modifying opioid peptide activity in the hippocampal formation. This is especially important to note because, clearly, the behaviors examined in these studies are not uniquely sensitive to hippocampal manipulations. Other sites within functionally related neural systems may serve as targets for the effects of opiate antagonist treatments. For example, such a target site may be provided by opioid peptides/binding sites located in the septal region.

TABLE 1. *Rats tested on the radial arm maze in novel spatial environments*

	Saline Vehicle Condition		Opiate Antagonist Condition	
N	Trials to Criterion	Errors to Criterion	Trials to Criterion	Errors to Crieterion
8	5.4	12.0	3.3*a	4.4*
10	4.9	12.2	3.6*b	5.8*
10	4.8	10.6	3.5*a	3.7*
27	5:8	12.0	3.7*c	5.0*

*Significantly different from vehicle.

[a]Drug treatment with naloxone (2 mg/kg).

[b]Drug treatment with diprenorphine (1 mg/kg).

[c]Drug treatment with naltrexone (1 mg/kg).

SOURCE: Adapted from Gallagher et al. 1983; Gallagher et al. 1985a.

OPIOID PEPTIDE REGULATION OF AFFERENTS TO THE HIPPOCAMPUS FORMATION

In a series of investigations dating from the late 1970's, micro-injections of agonists, morphine and opioid peptides, into the medial septal area (MSA) in rats were reported to inhibit ACh activity in the hippocampus (Botticelli and Wurtman 1982; Moroni et al. 1977; Moroni et al. 1978; Wood et al. 1979). For example, either morphine or beta-endorphin administration into the medial septum decreases ACh turnover in the hippocampal formation (Moroni et al. 1977). Inhibition of ACh by opioids is blocked by prior systemic administration of opiate antagonist, as well as by acute transection of the fimbria/fornix (Botticelli and Wurtman 1982; Moroni et al. 1977). Such findings led to the proposal that opiate-sensitive mechanisms in the vicinity of ACh perikarya inhibit septo-hippocampal ACh neural activity. Endogenous opioid peptides may also regulate normal septohippocampal ACh function. For example, systemic administration of naltrexone prevents inhibition of ACh activity in the hippocampus that can be induced by an episode of stress (Lai et al. 1988). It is notable that this effect of opiate antagonist treatment exhibits some selectivity within the basal forebrain ACh system. While the effect of stress on inhibition of

ACh activity in the hippocampus is prevented by naltrexone, the inhibition of ACh function induced in prefrontal cortex by the same behavioral manipulation remains unaffected by opiate antagonist treatment (Lai et al. 1988).

The MSA, which has served as a target site for opiate micro-injections, contains a relatively sparse innervation by opioid peptides. Dynorphin-like immunoreactivity is absent in the medial septal nucleus: scattered fibers are seen in the lateral septum (Khachaturian et al. 1982). Beta-endorphin innervation of the septal region appears to be confined to the ventral lateral septal nucleus in a region that does not encroach on the MSA (Finley et al. 1981). In a report by Gall and Moore (1984), perikarya exhibiting enkephalin-like immunoreactivity were found in the lateral septum, predominantly in the intermediate lateral septai nucleus. Enkephalin-immunoreactive fibers/terminals were observed in two zones: one located dorsolateral to the enkephalin cell bodies at rostral levels and midlevels of the septum, and another positioned along the lateral borders of the medial septal nucleus throughout its rostral/caudal extent.

The distribution of opioid peptides is interesting to consider alongside the pattern of opiate binding in the septum. Two zones of opiate-binding sites are apparent in autoradiograms of the septal region that correspond rather well with the localization of immunoreactive fibers/terminals reported by Gall and Moore (1984). In our analysis of autoradiograms generated from sections through the rostral/caudal extent of the region, this correspondence is apparent at all levels of the septum (unpublished observations). These autoradiograms of [^{3}H]naloxone binding show a pattern of distribution that is also similar to that depicted in a recent report using D-Ala^{2}-MePhe^{4}-Met(o)^{5}-ol-enkephalin (FK) to selectively label mu binding sites (McLean et al. 1988). In the present context, the distribution of opioid fibers/terminals and binding sites in proximity to the MSA is interesting, especially because this area may overlap the topography of choline acetyltransferase (ChAT)-immunoreactive neurons that project to the hippocampal formation. Following injection of retrograde tracer into the hippocampai formation, cells that double-labelled for ChAT immunoreactivity are notably absent along the midline (Amarai and Kurz 1984). Rather, these identified ACh projection neurons at the level of the septum are positioned laterally within the medial septai nucleus, and their distribution is

reported to extend Into the adjacent lateral septum. These anatomical details are consistent with the hypothesis that opiate-sensitive sites within the septal region are in a position to regulate septohippocampal ACh function.

Recent work in our laboratory has shown that microinjections of opiate treatments into the medial septum can alter performance on learning and memory tasks (Bostock and Gallagher, in press; Gallagher et al., in press). One testing procedure used in this work is the radial arm maze task already described. In an experiment in which the same animals were tested in four different spatial environments under vehicle and varying doses of naloxone, posttraining microinjection into the medial septum appreciably improved performance (table 2).

TABLE 2. *Effects of postraining naloxone administration into the medial septum on performance in new environments (n=10)*

Treatment	Trials to Criterion	Errors to Criterion
Vehicle	5.1 ± 0.4	14.2 ± 2.1
5 ng Naloxone	5.4 ± 0.4	13.5 ± 1.7
50 ng Naloxone	4.1 ± 0.4*	0.3 ± 1.8**
500 ng Naloxone	5.0 ± 0.4	9.0 ± 1.3*

*Different from vehicle treatment condition; correlated t test, $p<0.05$.

**Different from vehicle treatment condition: correlated t test, $p<0.01$.

NOTE: Values given are means ± SEM.

In related experiments (Bostock et al., in press), memory-based performance on this task was found to be impaired by medial septal administration of beta-endorphin over a range of doses previously shown to inhibit hippocampal ACh activity (5 to 500 ng) (table 3). As shown in table 3, the effects of beta-endorphin microinjections into this area have no detrimental effect on behavior, however, once animals have already learned to perform well in a given

spatial environment. These results suggest that opiate-sensitive sites located outside the hippocampal trisynaptic circuit alter the ability to retain new spatial information. However, these effects may be due, at least in part, to changes in hippocampal function via regulation of septohippocampal activity.

TABLE 3. *Effects of beta-endorphin on maze performance*

Treatment	Errors to Achieve Criterion[a]	Errors at Criterion[b]
Vehicle	13.9 ± 2.0	1.3±0.17
Beta-endorphin	32.0 ± 6.3*	0.9 ± 0.27

*Significantly different from vehicle, $p<0.05$.

[a]These animals (n=12) received vehicle or peptide (500 ng) on the first 4 days of testing in a new spatial environment.

[b]These animals (n=9) received vehicle or peptide (500 ng) on 4 consecutive days after reaching criterion in a spatial environment.

NOTE: Values given are means ± SEM.

Although opiate antagonists have proved to be quite effective in altering behavioral capacities that are associated with hippocampal function, the hippocampus per se is not necessarily a target for these effects. In this context, it may be important to note that other afferents to the hippocampal formation, such as the ascending noradrenergic system, may also be under opioid peptide control. Here, in contrast to the interactions described between septo-hippocampal ACh neurons and opiate-sensitive mechanisms, regulation of the norepinephrine (NE) system may occur at the level of both the NE cell bodies and the NE terminals (Montel et al. 1974; Nakamura et al. 1982; Pickel et al. 1979; Williams and North 1964). Inhibition of the evoked release of NE by opiate agonists has most recently been reported in hippocampal slices from several species including rats (Jackisch et al. 1984; Jackisch et al. 1966). Recent behavioral studies have shown that intact function in the dorsal noradrenergic pathway is necessary for obtaining the memory-enhancing effects of opiate antagonists (Fanelli et al. 1965;

Gallagher et al. 1985b). Thus, removing the NE system from opioid peptide inhibition at the level of the locus coeruleus (LC) and/or in terminal fields of the hippocampal formation may also influence these pharmacological effects.

OPIOID PEPTIDES IN THE HIPPOCAMPAL FORMATION

Turning to the opioid peptides that are localized within the hippocampal formation, there is little known about the conditions under which these opioid peptides are normally activated or the effects they exert on behavior. One interesting correlation has emerged recently between the effects of treatments aimed at blocking opioid peptide activity, including anti-Met-enkephalin antiserum administered into the hippocampus, and behavior in two different strains of mice (Castellano 1961; Van Abeelen and Gerads 1966; Van Abeelen and Van den Heuvel 1962). The DBA/2 and C57BL/6 mice exhibit opposing responses to each of the treatments used. For example, the DBA/2 mice, like rats, show increased retention after posttraining administration of naloxone. This same strain shows increased measures of exploration in a novel environment after intrahippocampal injection of either naloxone or Met-enkephalin antiserum. This finding is also reminiscent of the effects of systemic naloxone administration in rats, where increases in stimulus-directed behavior in a novel complex environment are found (Arnsten and Segal 1979). In contrast, the C57BL/6 mice yield an opposite pattern of results on each of these measures. These studies suggest that hippocampal opioid peptides are activated when mice explore a novel environment, but there are quite distinctive effects on behavior in these two genotypes.

Using a somewhat different approach, we have begun to examine whether exposing animals to training experiences that should engage hippocampal neurons will alter opioid peptide activity in rats. In this work, we have an additional interest in determining whether hippocampal inputs such as those arising from noradrenergic and cholinergic neurons can be shown to regulate opioid peptide activity. This interest arises from our focus on learning and memory, as well as from previous work reporting that lesions of the fimbria/fornix alter levels of hippocampal opioid peptides (Hoffman 1964; Hoffman and Zamir 1964; Hong and Schmid 1961).

Initially, we used classical conditioning procedures to examine whether exposing animals to training would alter opioid peptide

activity within the hippocampus (Meagher et al. 1966). There is substantial evidence that hippocampal neural activity is engaged during such associative learning. When classical conditioning procedures are used, conditioned responses are observed in a large proportion of pyramidal neurons In the hippocampus of rabbits (Berger and Thompson 1976). There is also evidence for neural correlates of associative learning in the dentate gyrus, as well as in the hippocampus, of rats (Deadwyier et al. 1979; Deiacour 1984; Rose 1983; Segal 1973; Segai et al. 1972). In our initial experiment, the animals that served as subjects received unilateral lesions of the dorsal noradrenergic bundle 3 to 4 weeks prior to the experiment. These rats were sacrificed either 30 minutes or 3 hours after a conditioning session in which a tone conditioned stimulus (CS) and footshock unconditioned stimulus (UCS) were either contingently presented (paired groups, 10 trials) or noncontingently presented (unpaired groups, 10 presentations of each stimulus). Subsequent radioimmunoassay for dynorphin A(1-8) in the hippocampal formation revealed a significant effect of training and of time of sacrifice but no effect of the NE lesion. Whereas dynorphin A(1-8)-like immunoreactivity (dynA(1-8) LI) was comparable for paired and unpaired groups sacrificed 3 hours after training (expressed as picomoies per gram of tissue, wet weight, 10.61 ± 0.41 and 9.64 ± 0.61, respectively), levels were significantly higher in the paired group relative to the unpaired group when rats were sacrificed 30 minutes after training (14.36 ± 0.61 and 10.36 ± 1.01, respectively). Radioimmunoassay of Met^5-enkephalin LI activity yielded no significant difference among these same treatment conditions.

These results indicate that training procedures similar to those that differentially affect the develorment of conditioned neural activity in the hippocampai formation may have distinctive effects on the content of dynA(1-8) LI presumably within the granule ceil/mossy fiber pathway in the hippocampus. The relatively higher level of dynA(1-6) LI in animals that received paired CS/UCS presentations might conceivably reflect lower utilization of opioid peptide relative to those animals that received shock noncontingently. Although unilateral NE lesions were not found to have a significant impact on these results, we are investigating the possible effects of bilateral NE denervation on opioid peptides. This approach may be particularly important when considering peptides associated with the granule cell/mossy fiber pathway because the dentate, as compared

to the hippocampus, receives a significant bilateral NE innervation (Loy et al. 1960).

Another condition that engages the activity of large populations of hippocampai neurons is found when rats explore their environments: hippocampai neurons have place fields that depend on the spatial location of the animal (O'Keefe and Nadei 1976). In addition, Sharpe et al. (1965) have found that exposing rats to a new spatial environment facilitates neural responses evoked in the perforant/ dentate pathway. This neural enhancement grows over several days' exposure to a complex spatial environment. The registration of spatial information in the activity of the hippocampal formation is particularly interesting because, as previously cited, lesions of this structure profoundly affect the ability of rats to perform accurately on spatial tasks.

in another preliminary study, however, we have not found any reliable effect on the opioid peptide content of the hippocampai formation when rats are trained on a spatial learning task. in this study, Long-Evans rats were trained for 4 days (four trials per day) to locate a camouflaged escape platform in a Morris water maze. These place-trained animals and their yoked controls, who spent an equivalent amount of time swimming in a tank, were sacrificed 15 minutes after completion of their last session along with experimentally naive subjects. Levels of dynA(1-8) LI and Met^5-enkephaiin LI in the hippocampus were not significantly different for animals across these conditions. This negative finding is somewhat more interesting when it is considered that hippocampai choiinergic function was affected in these same subjects. The contralateral hippocampus was used to measure sodium-dependent high-affinity choline uptake in these rats. This measure of ACh function in the hippocampai formation was selectively decreased in these same animals by place training relative to their yoked controls (Decker et al., in press). Thus, we have not yet found a functional relationship between a marker for septohippocampai ACh activity and altered levels of hippocampai Met^5-enkephalin LI, in contrast to the relationship found in previous reports that fimbria/ fornix transections elevate Met^5-enkephalin LI in the hippocampus (Hoffman 1964; Hoffman and Zamir 1964; Hong and Schmid 1961). Additionally, plasma corticosterone was measured in our rats at the time of sacrifice. Whereas a training-induced elevation in 11-hydroxycorticosteroid was observed relative to behaviorally naive rats, this index of pituitary-adrenal activation was not accompanied

by altered opioid peptide immunoreactivity in the hippocampai formation.

CONCLUSION

Although opiate antagonists are found to improve behaviors that are highly sensitive to hippocampal damage, we have cautioned that opiate-sensitive sites outside the hippocampus may be involved. Possible routes for opiate antagonist improvement of learning and memory include interactions with the basal forebrain choiinergic and ascending noradrenergic systems, both of which provide afferents to the hippocampai formation.

As research on opioid peptides enters its second decade, we are just on the threshold of exploring the functional properties of these neuropeptides in the hippocampai formation. No conclusions can yet be drawn about their biological significance. At present, we know almost nothing about the conditions that favor their release or the physiological responses evoked by endogenous opioid peptides-ones that might be revealed in the presence of appropriate antagonists. The view expressed here is that currently available information about natural stimuli that engage hippocampal neural activity in intact animals may prove a useful and necessary adjunct to research using *in vitro* preparations.

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AUTHOR

Michela Gallagher, Ph.D.
Department of Psychology
University of North Carolina
at Chapel Hill
Davie Hall 013A
Chapel Hill, NC 27514

Effects of Mu Opioid Receptor Activation in Rat Hippocampal Slice

Menahem Segal

INTRODUCTION

In the hippocampus, the major action of opioid peptides is to enhance excitability of both pyramidal and granule cells (Corrigall 1983; Henderson 1983; Zieglgänsberger et al. 1979). Although this fundamental observation is now generally accepted as true, our knowledge of opioid action even at this simple level of description is still incomplete. The specific actions of different classes of opioids in each region of the hippocampus, for example, have not yet been systematically studied. Most electrophysiological experiments have been conducted in the CA1 region of the dorsal hippocampus, where recording is easiest; yet there are, in the adjacent CA2 region, three times as many mu- and delta-binding sites as in CA1 (Crain et al. 1986; Herkenham and McLean, this volume). Similarly, the ventral hippocampus is far richer in opioid peptides than the dorsal hippocampus.

The opioid-induced excitation of pyramidal and granule cells is reportedly due to disinhibition resulting from the hyperpolarization of inhibitory gamma-aminobutyric acid (GABA)-containing interneurons (Nicoll et al. 1980; Zieglgänsberger et al. 1979). The evidence for this relies on occasional recording from interneurons in hippocampal slices (Nicoll et al. 1980) and is also supported by *in vitro* experiments from other brain structures. In the nucleus locus coeruleus, particularly, it has been shown convincingly that opioids cause an increase in some types of potassium currents, which act to decrease neural excitability (Williams et al. 1982). In other systems, opioid peptides are found to cause other effects, perhaps on calcium currents (Werz and Macdonald 1982).

Whether or not disinhibition is the only mechanism that mediates opiate action on hippocampal pyramidal ceils and dentate gyrus granule cells is not clear; there is evidence that opioid peptides may enhance excitatory postsynaptic potentials (EPSPs) directly, even when inhibitory postsynaptic potentials (IPSPs) are not affected by these drugs (Dingledine 1981; Gähwiler 1983; Haas and Ryall 1981; Lynch et al. 1981). Furthermore, it is not clear that excitation is the only effect of opiold peptides on hippocampal pyramidal ceils; there have been several reports of inhibitory actions of some peptides and opioid analogues on hippocampal activity (Henriksen et al. 1982; lwama et al. 1986; Moises and Walker 1985). Clearly, additional work is still needed to sort out the differences among conflicting reports. The use of selective agonists for mu, delta, and kappa receptors (Bostock et al. 1984; Brookes and Bradley 1984; Chavkin et al. 1985; Neumaier and Chavkin 1986; Henderson 1983; lwama et al. 1986; Tielen et al. 1981; Valentino and Dingledine 1982; Vidal et al. 1984) will certainly facilitate classification of different response types, although particular caution must be exercised in using ligands in the proper concentration range, especially when only a relative selectivity has been demonstrated.

Another puzzling observation is the lack of evidence of any opioid role following activation of the mossy fiber system (the pathway that connects the dentate gyrus to the hippocampus proper) which is known to contain dynorphin A. No naloxone-sensitive response is seen in CA3 neurons following stimulation of this pathway (Chavkin and Bloom 1986; Moises and Walker 1985; Henriksen et al., this volume). Because the mossy fiber system is presumed to contain an excitatory amino acid (EAA) neurotransmitter in addition to dynorphin A, a potential role of the opioids in the hippocampus may be in modulating the effects of other transmitters (Segal 1977). There is already evidence that mu, delta, and kappa agonists can modulate the release of acetylcholine or norepinephrine from hippocampal slices (Jackisch et al. 1986). Moreover, in the hippocampus, the enzyme acetylcholinesterase, which is responsible for the rapid breakdown of acetylcholine, is not only colocalized with opiate receptors but also has peptidase activity of its own. It may therefore be involved in the inactivation of opioid peptide activity in places where acetylcholine and opioids overlap.

Opioid peptides may also modulate EAA-induced neurotoxicity. Injection of kainic acid into the hippocampus, the cerebral ventricles,

or parenterally, produces marked and specific changes in the hippocampus involving initial seizure activity followed by neuronal death. Concurrent with these seizures are marked initial reductions and later increases in opioid peptide concentrations in the hippocampus (Kanamatsu et al. 1986, Gall, this volume). Interestingly, region CA2, the region with the highest concentration of opioid-binding sites, is also the region most resistant to the toxic effects of kainic acid. Although it still has to be shown that the intrinsic opioids play a role in protecting CA2 from the destructive effects of EAA neurotoxins, this finding is consistent with such an effect.

Most importantly, the functional significance of opioid peptides in the normal operation of the hippocampus remains elusive. In part, the problem may be divided according to the two main hippocampal peptide types. If dentate gyrus granule cells do indeed release dynorphin from terminals as a neurotransmitter or cotransmitter, and if enkephalin is similarly released by the terminals of perforant path fibers originating in the entorhinal cortex, then correlation of the physiological activity of these classes of neurons with the effects on their targets should yield information on the involvement of these peptides in the hippocampus. To date, however, it has proved quite difficult to record from identified opioid cells. As a result, most information concerning opioid involvement in hippocampal function has been accumulated by applying agonists and antagonists to the postsynaptic targets of the mossy fiber and perforant pathway systems. Some assessment of opioid concentration in the hippocampus during structured behavioral tests have also been utilized to analyze opioid actions on learning (Gallagher, this volume).

The experiments described below address some of the questions raised here, and the findings are used to propose a mode of action for and possible function of the opioid peptides acting at mu receptors in the hippocampus. Specifically, the findings address the possibility of differences in excitability between regions CA1 and CA2 in response to the application of the mu agonist D-Ala^2-MePhe^4-Gly-ol^5-enkephalin (DAGO) (Jackisch et al. 1986), differences in opioid action along the septotemporal axis of the hippocampus, the involvement of other neurotransmitters and extrinsic afferents in the observed effects of opioids in the hippocampus, and some of the functional implications of activating mu receptors in the hippocampus.

METHODS

Experiments were conducted with transverse slices of rat hippocampus as described elsewhere (Segal 1986). An intracellular recording was made with glass micropipettes containing K-acetate, and an extracellular recording was made with low-resistance (2- to 3-M) micropipettes containing the superfusion medium. Signals were amplified with an Axoclamp II amplifier and recorded continuously on a chart recorder. Drugs were applied with the microdrop technique on the slice, near the recording site. Only one cell (or extracellular recording position) was recorded from a given slice. DAGO (a gift from Dr. R. Simantov of the Weizmann Institute, Rehovot, Israel) was applied at a microdrop concentration of 1 μM; the final concentration at the receptor site is unknown but is probably 10 to 100 times smaller than at the microdrop.

REGIONAL EFFECTS OF DAGO

The first series of experiments was conducted in order to compare reactivity of CA1 and CA2 neurons to topical application of DAGO. Systematic experiments were done with eight CA1 and seven CA2 neurons. Resting membrane potential (range -65 to -80 mV) and spike height (80 to 110 mV) were not different between the two cell groups. The Input resistance of CA2 cells was slightly lower than that of CA1 cells (range 12 to 60 mohm). CA2 ceils possess a unique rectifytng property that was not found in CA1 neurons (Segal, in preparation). Topical application of DAGO to CA1 cells produced no significant change in the resting properties of the recorded neuron. The only marked effect, seen by others with mu agonists in CA1 cells (Bostock et al. 1984), was a reduction in the two-component slow IPSP evoked in response to stimulation of the stratum radiatum. Concurrent with this, the initial EPSP evoked by the same stimulation was enhanced. In general, the EPSP slope was unchanged, indicating that the reduction in the IPSP size can account for the changes in EPSPs. Similar responses were detected in all CA2 cells tested, with no qualitative differences from the CA1 responses. in four of the seven CA2 cells examined, application of DAGO produced a mild (2 to 2.5 mV) hyperpolarization (figure 1). This response lasted 1 to 2 minutes and recovered, while the effect of DAGO on the EPSP-IPSP sequence recovered only 10 to 20 minutes after drug application. It appears that DAGO may have two independent effects on CA2 cells, only one of

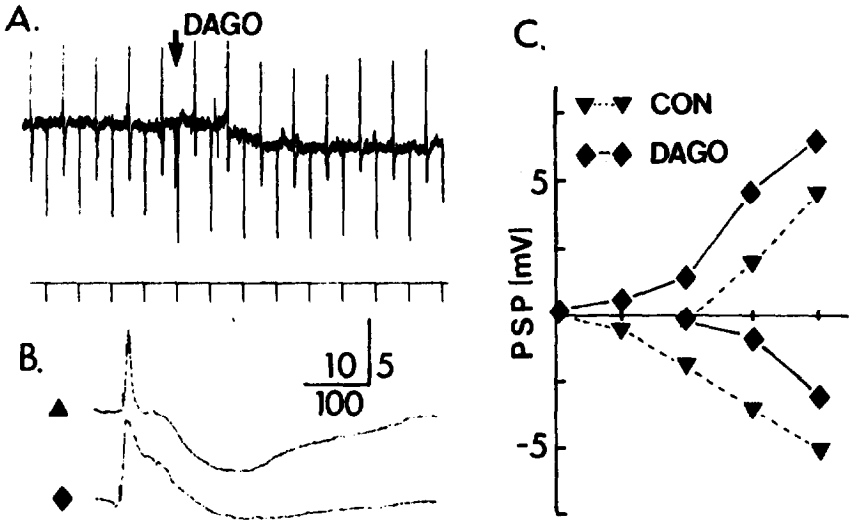

FIGURE 1. *Effects of DAGO on properties of a CA2 neuron recorded intracellularly*

NOTE: (A) Specimen record of membrane potential -65 mV) and responses to hyperpolarizing current pulses and afferent stimulation before and after a microdrop application of DAGO. The hyperpolarizing current pulses are applied every 5 seconds (bottom record current) and alternate with afferent stimulation. The latter produces a biphasic response consisting of an EPSP followed by a slow IPSP. DAGO produces a 2-mV slow hyperpolarization that develops over 15 seconds after drug application. (B) An illustration of the EPSP-IPBP sequence before (top) and after (bottom) application of DAGO. Time scale: 10 seconds for A, 100 msec for B, 5 mV for both. (C) Input-output relations of the size of the EPBP and IPSP as a function of simulation intensity before (control, CON) and after (DAGO) application of the drug.

which is shared by CA1 neurons. The pharmacological nature of the hyperpolarizing response has yet to be determined.

EXTRINSIC MODULATION OF REACTIVITY TO DAGO

Stimulation of the excitatory afferents residing in the stratum radlatum can generate a population action potential (population spike) in the recorded hippocampus. This population spike results from concurrent activation of many cells in the recorded area. Normally, a single population spike is generated and is followed by an

inhibitory period corresponding to the duration of the IPSP. Topical application of opiates has been shown by several investigators to produce a larger population spike, and this is commonly followed by a secondary afterdischarge (Corrigall and Linseman 1980). We could replicate these responses following application of DAGO (figure 2). A secondary afterdischarge, not seen normally in our preparation, amounted to 48 percent of the initial population spike after application of DAGO (n=12 slices).

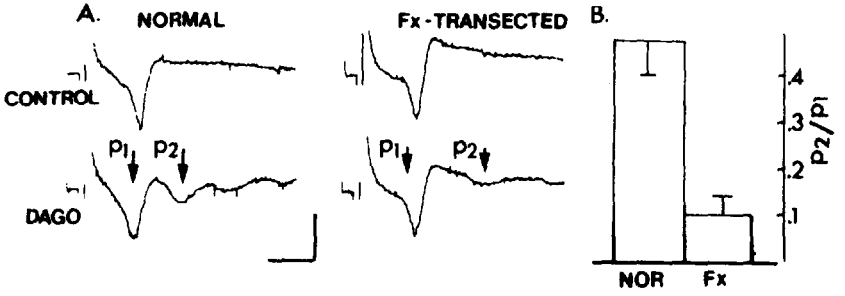

FIGURE 2. *Effect of prior fornix transection on the efficacy of DAGO-induced secondary afterpotential (P2)*

NOTE: (A) Examples shown are taken from two slices recorded in the same experiment, of normal and fornix (Fx)-transected rat hippocampi. Complete, bilateral Fx transection was performed 1 to 2 weeks before recording. This transection results in nearly total loss of hippocampal content of acetylcholinesterase. Neither of the two slices produce a secondary potential under the control condition, and both produce one in the presence of DAGO, but the ratio of the second to the first (P2/PI) is larger In the normal slice. (B) Data from 12 normal slices and 11 Fx-lesioned slices are averaged. Error bars are standard errors of the mean.

In one of several types of experiments designed to examine the modifiability of the response to DAGO, we used fornix-transected rats. Fornix transection depletes the hippocampus of most of its cholinergic, serotonergic, and noradrenergic afferents. In addition, it disrupts the feedback circuit between the hippocampus and the septal nuclei. It has also been reported that fornix transection, or septal lesion, produces a transient rise in concentrations of opioid

peptides in the hippocampus (Hong and Schmid 1981). In 11 slices taken from 4 fornix-transected rat hippocampi, we found that DAGO produced only a small secondary afterdischarge, about 10 percent of the initial population spike amplitude. These results suggest one of three possibillties: (1) That fornix transection reduces the ability to evoke an afterdischarge (this is unlikely since, in these same slices, a tetanic stimulation is capable of producing an afterdischarge (Segal, unpublished)); (2) that intrinsic opioid-containing neurons are regulated by extrinsic afferents, and the absence of these afferents causes increased activity of opioid-containing neurons, enhanced production of opioid peptides, and consequent subsensitivity of postsynaptic opiate receptors; and (3) that DAGO produces at least part of its observed effects by acting on afferent terminals arriving via the fornix, and in their absence the afterdischarge is not recorded. Further experiments are needed to examine these possibilities.

THE NATURE OF THE SYNAPSE ACTIVATED BY DAGO

The enhancement of EPSPs and the blockade of IPSPs result in the appearance of a secondary afterdischarge. This enhanced excitability can also result from activation on the postsynaptic neurons of additional receptors that are normally masked by the presence of inhibitors. It has been suggested that these additional voltage-dependent responses are mediated by activation of N-methyl-D-aspartate (NMDA)-like receptors (Collingridge et al. 1983). If that is the case, one should be able to block the afterdischarge by application of an NMDA receptor antagonist. Indeed, application of P-amino phosphorovaleric acid (2-APV) did block the afterdischarge while having only a small effect on the DAGO-enhanced primary population spike (figure 3).

The possibility that opioid peptides uncover a voltage-dependent NMDA-like receptor has fascinating implications; these receptors are probably involved in plastic properties of the hippocampus, including its ability to exhibit long-term potentiation (Kelso et al. 1986; Wigstrum and Gustafsson 1983). The effect of DAGO on the ability of hippocampal neurons to undergo long-term synaptic changes was examined in a test condition where synaptic input was associated with postsynaptic depolarization. A similar condition was considered recently to be an illustration of a Hebbian synapse (Kelso et al. 1986), that is, an excitatory synapse that, when activated when the cell was depolarized, demonstrated subsequent

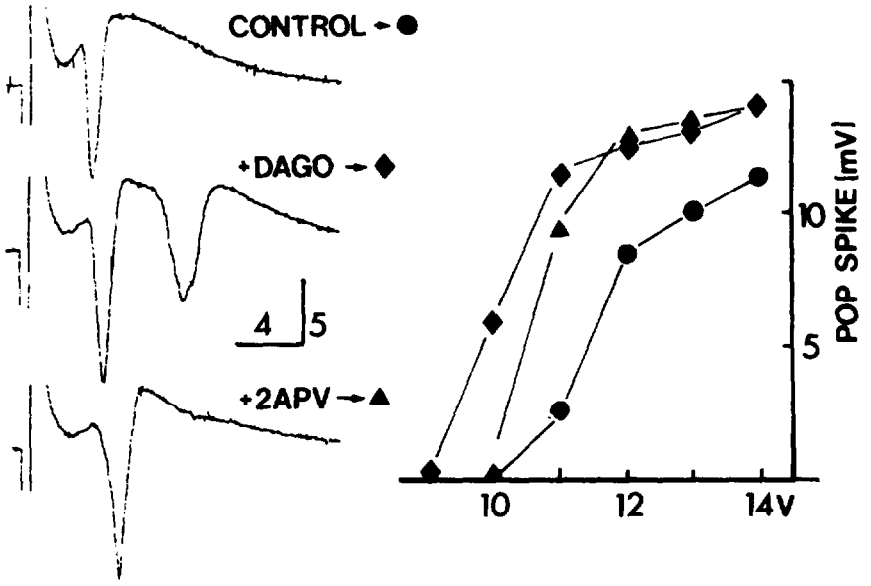

FIGURE 3. *Effects of NMDA antagonist 2-APV on a secondary population spike evoked in the presence of DAGO*

NOTE: On the left are specimen extracellular records taken (top to bottom) before and after application of DAGO. and after application of 2-APV in the presence of DAGO. Note the total disappearance of the secondary population spike in the presence of 2-APV. On the right Is an input-output curve of the size of the initial population spike under the three recording conditions (control, DAGO, and DAGO + 2-APV).

enhancement of its excitation. Initially, we stimulated the stratum radiatum to record a stable intracellular EPSP followed by the typical IPSP. The cell was then depolarized by passage of DC current pulse concurrent with the application of the afferent stimulation. Under these conditions, we were unable to detect subsequent long-term changes in the magnitude of the EPSPs elicited by stratum radiatum stimulation. We then applied DAGO (five cells) and found that subsequent association between depolarizing current pulses and EPSPs now produced long-term enhancement of the EPSPs (figure 4). This enabling function of DAGO might provide a clue to a role of endogenous opioid peptides in the hippocampus; activation of opioid neurons may enhance local plasticity. This possibility will be examined in future experiments.

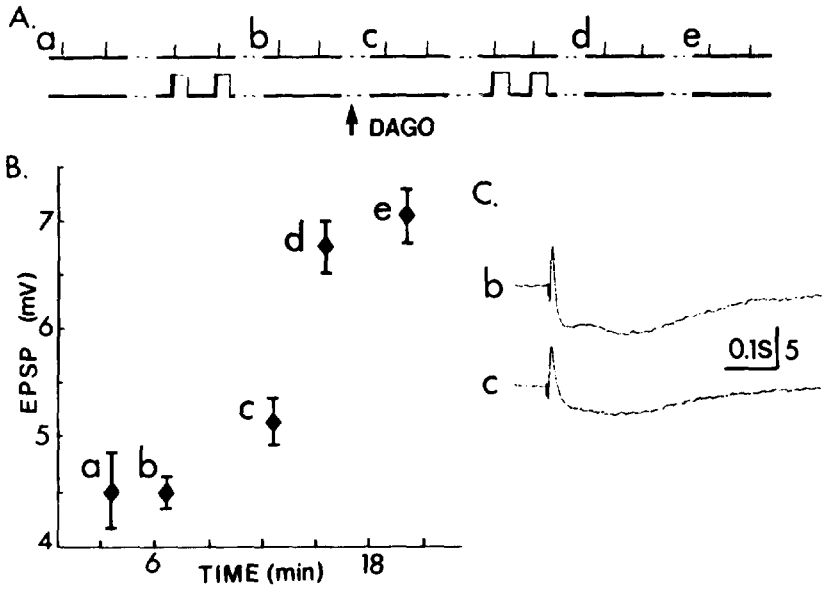

FIGURE 4. *Enabling action of DAGO on potentietion of EPSPs in a hippocampal neuron*

NOTE: (A) Schematic presentation of the stimulatlon paradigm: Top row is afferent stimulation; bottom row is intracellular current injection; a to e refer to averaged PSPs depicted in B. Afferent stimulation was applied once every 5 seconds. (a) Before association of the afferent stimulation with the depolarizing current pulses: (b) 1 to 5 minutes after a series of five large depolarizing current pulses, which produce a series of action potentials, applied simultaneously with the afferent stimulation; (c) 5 minutes after application of DAGO; (d) as in b; and (e) 10 minutes later. (B) Series of averages of 10 to 20 PSPs recorded in the conditions seen in A. (C) Individual PSPs on an expanded scale taken before (during period b) and after (period c) application of DAGO.

DISCUSSION

The present experiments address some puzzles accumulated over the past few years in the study of opioid peptides in the rat hippocampus. The apparent involvement of extrinsic afferents coming via the fomix-fimbria in the action of DAGO may indicate that, in order to appreciate the extent of opioid effects, one has to activate these extrinsic afferents.

This is difficult to achieve in the artificial situation inherent in the slice preparation, where the extrinsic afferents are not functional. Hence, the disinhibiiory action of DAGO and the other opioid peptides might reflect one of several cases of presynaptic action of these peptides on hippocampal circuits. The reduced efficacy of DAGO in the fornix-transected hippocampus is consistent with the reported effects of opioids on release of acetylcholine and noradrenaline in the hippocampus. Acetyicholine has several loci of action in the hippocampus and elsewhere: it causes a decrease in evoked PSPs, depolarizes postsynaptic cells in a slow manner, produces a fast activation of interneurons, and causes an increase in spontaneous PSPs. It is possible that the opioid peptides modulate only some of these effects, i.e., those taking place near the pyramidal cell layer. If this is indeed the case, it introduces a measure of specificity into a neural pathway-the cholinergic septohippocampal connection-which is inherently diffuse.

The enabling action of DAGO on activation of an NMDA receptor is of important functional significance. In previous experiments on a Hebbian synapse, it was necessary to block inhibition using GABA antagonists (Kelso et al. 1986). The meaning of such experiments was not clear because the brain is not known to contain an intrinsic GABA-antagonist-like compound. If an opioid-containing neuron can function to enable an NMDA receptor, then the interpretation of such experiments is more straightforward. One has to find the conditions that will activate opiold-containing neurons and demonstrate that such conditions will also facilitate plasticity in the hippocampus and then learning by the animal.

SUMMARY

The effects of the mu opioid agonist DAGO were examined in the hippocampal slice preparation. The effects of the drug on active and passive properties of neurons in CA1 and CA2 were examined. Intracellular responses of these neurons to afferent stimulation were examined before and after DAGO application. As seen with other mu agonists, DAGO reduced IPSPs and enhanced the duration of EPSPs but not their rise time. in CA2 cells, DAGO also produced a transient hyperpolarization. Further experiments examined the interactions of the opioid peptides with other neurotransmitter systems in the hippocampus. The effects of DAGO were reduced by prior fornix transection. Some effects of DAGO on generation of a

secondary afterdischarge are abolished by an NMDA receptor antagonist, 2-APV. The significance of these results to the understanding of functioning of the hippocampal circuit is discussed.

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AUTHOR

Menahem Segal, Ph.D.
Center for Neurosciences
Weizmann institute of Science
Rehovot 76100, Israel

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