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Opiate Receptor Subtypes and Brain Function

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Opiate Receptor Subtypes and Brain Function

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Opiate Receptor Subtypes and Brain Function

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Contents

Preface

OPIOID RECEPTOR SUBTYPES AND BRAIN FUNCTIDN

A technical review was held on September 26 and 27, 1985, at the National Institute on Drug Abuse to examine the current state of knowledge of opiate receptor subtypes and specific opioid peptides that act on these receptor subtypes, and to consider how brain function might be related to the diversity of receptor/ligand combinations. This monograph consists of papers delivered at this review. The conclusion of each presentation, suggested or expressed, was that there are indeed a variety of opioid receptors differing in ligand specificity and location in the nervous system, and that these receptors do mediate different functions. The current descriptions of the complex processes underlying the functioning of opioid neuronal pathways have been derived from investigations at all levels from the molecular to the behavioral. It now appears that by combining new neuroscience technology with well-described behavioral paradigms, questions about the effects of the various peptide-ligands and receptors on behavior itself can be answered. As a group, the papers reveal the widespread distribution and influence of opiopeptides within the nervous system and highlight some of the exquisitely sensitive control mechanisms that allow these systems to react to and counteract the varieties of influences that make up our daily lives as well as those of drug abusers.

Multiple Receptor Subtypes Defined

The uses of the various methods that serve to define opioid receptors and pitfalls of these methods were described by BRIAN COX. The original definition of subtypes termed the mu, kappa, and sigma receptors was based on physiological responses to opiates in the chronic spinal dog. More recently, two new subtypes, delta and epsilon receptors, have been recognized as well. Newer methodologies for characterizing receptors have utilized biochemical measures of binding affinities (K_D), total numbers of receptors (B_{max}), potencies of various agonists (A_{50} and IC_{50}), agonist affinities as measured from agonist effects? (K_A), and estimates of

antagonist affinity (K_e). Although a few of the endogenous opioid ligands may have exquisite specificity for some subtypes of the opiate receptor, the synthetic narcotic agonists and antagonists and the opiopeptide-related synthetic compounds generally react with more than one subtype of opiate receptor. Although the specificity of synthetic ligands continues to improve, cross-reactivity makes the determination of the physical characteristics of the receptors difficult.

Many neuropeptide hormones exist as large molecules, sometimes active, that can be hydrolyzed by peptidase action into smaller peptides that are active harmonally. Proopiomelanocortin is such a peptide. It is processed into several active subunits including beta-endorphin. In turn, beta-endorphin is hydrolyzed into smaller subunits including methionine-enkephalin. Dynorphin is another opiopeptide that can be hydrolized into smaller active peptides. NANCY LEE described this process, showing that short peptides tended to react with mu and delta receptors, while longer segments reacted at kappa receptors. Using refined methodology, she showed that none of the dynorphin subunits seem to have a clear specificity for any one receptor subtype.

SUZANNE ZUKIN summarized our knowledge of opioid receptor subtypes (table 1), opioid receptor subtype distribution in brain, and ligand specificities. In addition to biochemically defined receptor properties, the dynamic nature of the receptors is emphasized; they can change in number or affinity depending on the physiological state or previous drug history. An example of receptor dynamics is "up-regulation" in response to opiate antagonist administration. Treatment with an opiate antagonist such as naloxone results in (1) a supersensitivity to morphine, (2) an increase in mu receptor density observed in layers I and I II of frontal cortex, nucleus accumbens, and amygdala, and (3) increased enkephalin levels. This up-regulation is not permanent but requires about 6 days to return to predrug status.

Opioid receptor subtypes were further defined and classified by PAUL WOOD from the perspective of Martin's 1976 definition of mu, kappa, and sigma receptors, the receptor bindinq properties of mu and delta ligands, and the *in vitro* immunological characterization of mu, delta, and epsilon subtypes. He further categorized opiate subtypes into mu_1 and mu_2 as well as $kappa_1$ and $kappa_2$ receptors. Since kappa agonists act as antagonists at the mu_2 receptor, it may be possible to either develop an analgesic that has less unwanted side effects, or to combine kappa and mu agonists to obtain analgesic effects while blocking out the unwanted side effects.

The Neuron Model

Anatomical and biochemical perspectives of opioid peptide and opiate receptor subtypes were presented by ROBERT ELDE, who discussed our current understandins of opioid function in terms of the neuron model. The classic criteria for identifying transmitters are: (1) distribution, (2) production within the neuron,

TABLE 1. *Receptor subtypes and ligand specificity*

Receptor	Agonists	Antagonists	Physiological Actions
Mu_1	Normorphine Morphiceptin DAGO	Naloxone Naloxonazine Beta-FNA	Analgesia, temperature control
Mu_2	Morphine Sufentanil DAGO	U-50,488H Ethylketocyclazocine Naloxone	Respiratory depression, GH release, constipation
Delta	Enkephalins DPDPE, DADLE	ICI-174,864 ICI-154,129	Euphoria, brain reward
$Kappa_1$	U-50,488H Dynorphin (1-11)		Stimulation, locomotor activity
$Kappa_2$	Metenkeph.arg.phe Beta-endorphin Et.ketocyclazocine		Ataxia
Epsilon	Beta-endorphin		
Sigma	PCP, 3-PPP Pentazocine (+)SKF-10,047 (high affinity)	Haloperidol	Psychotomimetic effects
PCP	PCP, TCP Ketamine (+)SKF-10,047 (low affinity)		Sedation, stupor, psychotomimetic effects

NOTE: The chemical names of the compounds designated by letters are found in the text.

(3) release and inactivation, (4) identity of action, and (5) antagonism. It is now clear that opioids and their receptors have a selective, though widespread distribution. The peptides are manufactured from large propeptides, which may contain a variety of products. Activation, as well as inactivation, occurs by proteolytic cleavage. The difficulty in establishing a good neuron model for the Dpioid system is the same problem that has caused difficulties with other neurotransmitters; that is, the diffuse nature of peptide distribution and the difficulty of finding a brain region with a chemically homogeneous neuron population to examine closely. Another problem lies in the specificity of drugs available to study these systems. For example, although antagonists to most receptor subtypes have been found, the usual difficulty ensues: the antagonists are not specific for the subtype. The so-called "mismatch" of opioid receptors with peptidergic nerve terminals was also discussed. In some brain regions, there are high concentrations of opioid peptides but few receptors, whereas in other regions there are many receptors but few peptides. This mismatch may be more apparent than real for several reasons. The release of a ligand in amounts considerably less than the amounts of receptor would be a very sensitive way of producing short-term regulation. On the other hand, low receptor

population with high ligand levels might create a long-term regulation of neurotransmitter release at the synapse. In addition, it is also possible that, since histochemical identification of opiopeptides usually involves antibody interaction, the same antibody may not recognize peptide fragments that are not completely processed, thus underestimating the amount of ligand.

Transmitter Release Utilization, and Receptor Activation

One approach to relate opioid receptor function to ligands that bind to the opioid receptors is to examine the role of second messenger mechanisms in opioid action. STEVEN CHILDERS described the pharmacology of the well-known ability of opiates to cause physiological inhibition of adenylate cyclase. Activation of adenyl cyclase requires the binding of the activating transmitter to a receptor that is linked to an N(+) protein which, in turn, is linked to the cyclase itself. Inhibition of adenylate cyclase occurs through a similar mechanism, except that the cyclase link is a N(-) protein. However, when a striatal tissue preparation was exposed to low pH that uncouples the N(-) protein, various opioid ligands still inhibited adenylate cyclase activity, suggesting that an as yet undiscovered mechanism is responsible for the inhibition.

R. ALAN NORTH described changes in voltages and currents induced by altered flow across nerve cell membranes that result from activation of specific subtypes of opiate receptors. For these studies, a variety of nervous tissues were used including the submucous plexus of the guinea pig ileum, which has a pure population of delta receptors, and the locus coeruleus, which contains only mu receptors. In all cases studied so far, activation of opioid receptors leads to inhibition of the neuronal firing. The ionic events which take place during inhibition include an efflux of K^+ that hyperpolarizes the cell and reduces Ca^{++} influx. Reduced Ca influx reduces the amount of transmitter released even if the cell continues to fire. An inhibition of firing in the ileum is produced by delta receptor activation, whereas mu agonists have the same effect in the locus coeruleus. Both receptors seem to be linked to the same kind of K^+ channel. Selective delta and mu receptor activation decrease Ca^{++} transport as a result of K^+ conductance increases, whereas selective kappa receptor activation in the neurons of the myenteric plexus appears to decrease directly the entry of Ca^{++} into these cells. Thus, opioids may inhibit neural activity and transmitter release by a variety of mechanisms, some of which are also shared by nonopioid transmitters such as norepinephrine.

JACQUELINE MCGINTY has developed models to study opioid metabolism. release. and utilization. She has studied alterations in these parameters in the hippocampus following electroconvulsive seizures. The excitatory state of neurons induced by the convulsions is correlated with an initial loss of enkephalin, then a subsequent rebound in the content of this peptide within the neurons. In addition, there is an increase in mRNA for enkephalin

biosynthesis which is concomitant with enkephalin release, increased levels of intermediary enkephalin products, and eventually, increased levels of enkephalin. Whereas immunostaining shows a quick increase in met-enkephalin levels after seizures, it shows only a slow return to baseline for dynorphin levels, which are also lowered during seizures. This suggests that the loss of dynorphin does not lead to a stimulation of prohormone biosynthesis or processing in this paradigm.

Behavioral Expression of Opioid Receptor Activation

Behavioral models which distinguish opioid receptor subtypes were described by four neuropsychopharmacologists. STEVEN HOLTZMAN described a "psychophysics" approach to determining selectivity of opioid receptor activation using the paradigm of stimulus discrimination. An animal is trained to press one of two levers for food or water under the influence of the "training" drug (kappa agonist, for example) and to press the other lever following an injection of vehicle. When the discrimination ("drug" vs. "no drug") is established, the ability of other drugs to generalize to the training drug is tested. Using this approach, the ability of an opiate to exert a behavioral effect on a particular receptor subtype is evaluated using one or all of the following criteria: (1) similarity of effect, (2) stereospecificity, (3) specificity of antagonist actions, and (4) order of potency. When morphine (a mu agonist) was used as the training drug, etonitazine was found to strongly mimic morphine action, while codeine or propoxyphene did not generalize to morphine until they were given in high doses. Presumably, mu agonism is weak in the latter two drugs, and high doses are needed before a threshold mu effect is obtained. Other receptor agonists can be used as the basis for discrimination. PCP was found to generalize to sigma agonists (SKF-10,047, cyclazocine, ketamine, or dextrorphan). That the locus of the discrimination is central is shown by the demonstration that the behavior can be established following intraventricular as well as systemic administration.

An approach to studying the neurobiological basis of drug-seeking behavior was presented by JAMES E. SMITH who has found, using a yoked-control procedure, that the biochemical consequences of active drug self-administration are different from those resulting from passive drug infusion. Further, a role for the ventral tegmental dopamine system (the A10 cell group and its projections) has been established in mediating the reinforcing action of a variety of drug classes, including opiates. The nucleus accumbens, a projection area of the A10 cell group, contains primarily delta opiate receptors, whereas the ventral tegmental region itself contains mostly mu receptors. The behavioral relevancy of this receptor distribution is that a low to moderate dose of morphine is self-administered into the ventral tegmental area (VTA) but not into the nucleus accumbens, but a high dose is administered into both regions. Presumably it takes high doses of morphine, which is primarily a mu agonist, to activate delta receptors. Consistent with this differential activation of opioid

receptor subtypes in the brain is the finding that met-enkephalin, a delta agonist, is self-administered into the nucleus accumbens at low doses. Thus, at least two opiate receptor subtypes and the neural systems with which they are associated play an important role in the reinforcing action of drugs.

The use of a place-preference paradigm to evaluate the reinforcing qualities of kappa, mu, and other opiate receptor agonists was discussed by GEORGE KOOB. In this model, an animal-is placed in one side of a two-compartment chamber and injected with a test drug. The tendency to return to the location where drug was previously received is correlated with the drug's reinforcing qualities. In general, kappa agonists were not found to be particularly reinforcing in this system. On the other hand, beta-endorphin was found to be reinforcing at low doses. A variety of experiments have implicated the delta receptor, in addition to the mu and epsilon receptors, in the rewarding action of this peptide. The lack of place preference at high doses was due to the induction of a state of catatonia which is incompatible with place-preference behavior.

A fourth approach to testing the functional role of ooioid receptor activation was discussed by ROY WISE, who has discovered that a neural network involved in feeding behavior can be activated in a frequency-dependent manner by stimulation of either the ventral tegmental area (VTA) or the periaqueductal gray (PAG) region. In VTA neurons, the activation threshold is lowered by D-Pen-D-Pen enkephalin (or DPDPE, a delta agonist), morphine (a mu agonist), or U-50,488H (a kappa agonist), whereas in the PAG the delta and kappa agonists had no effect, but morphine still facilitated the behavior. This is a new behavioral paradigm that has already shown its utility in evaluating the action of opioid receptor subtypes, and this approach might be used to examine the hypothesis that several reinforcers may all activate a common pathway.

Clinical Aspects

The search for a nonaddicting narcotic analgesic has led us to discover opioid receptors and endogenous opiatelike substances in the brain. The descriptions of several subtypes of the opiate receptor and of drugs which can selectively stimulate these sites have brought the search for selective analgesia closer to its goal than ever before. In the process, anatomical and/or biochemical dissociation of the substrates underlying analgesia, physical dependence, and euphoria has been demonstrated. In addition, the functions of a number of opiate receptors have come to light and a number of research programs are actively exploring ways to use this information clinically. Some of the avenues being explored in relation to drugs with specific action on receptor subtypes were described by JOHN HOLADAY, who discussed the roles of opiate receptor subtypes in a of clinical conditions:

(1) Kappa receptors and dynorphins appear to play a role in regulating the severity of both spinal injury due to trauma and

any central nervous system (CNS) damage due to ischemia. The evidence is that the spinal cord contains large numbers of kappa receptors, dynorphin levels increase in spinal injury, and dynorphin can induce a paralysis that is reversed by kappa antagonists. Thus, kappa antagonists might be useful in treating ischemia and stroke.

(2) Opiate receptors are also involved in some forms of shock. Naloxone reverses endotoxin-induced hypotension, and this may be due to an involvement of delta receptors. The hypotension occurring in shock may be mediated by delta receptors, because delta agonists produce hypotension and delta antagonists can block the shock-induced hypotension.

(3) Mu and delta receptors may modify seizures. The flurothyl-induced seizure model has been studied using a variety of selective ligands. Etorphine potently elevated seizure threshold, an effect that was totally blocked by mu antagonists but only partially blocked by delta antagonists. Delta antagonists entirely blocked the ability of DADL to elevate seizure thresholds, whereas mu antagonists had no effect on DADL. This model has also produced interesting data suggesting that some kappa agonists may act also as longlasting mu antagonists and as antagonists to delta antagonists. These findings suggest interesting interactions among the subtypes of opiate receptors and may be interpreted as indicating close anatomical linkage between at least the mu and delta receptors, a suggestion that finds support from other findings as well.

Summary

During the past decade, we have made impressive theoretical and practical progress in defining opioid systems and relating specific opioid peptides and specific subtypes of opiate receptors to specific behaviors. Table 1 presents a summary of current knowledge of the relationship of receptor subtypes to behavior. During the next decade, we will see not only the full development of the neuron model, but our understanding of the role of opioids in brain function will increase dramatically. We also anticipate a host of new opioid-based pharmaceuticals and new modes of treatment not only for drug-abuse-related conditions, but, as suggested by Dr. Holaday's presentation, for conditions that now have no conceivable relation to drug abuse.

THE EDITORS

Properties of Receptors Mediating Opioid Effects: Discrimination of Receptor Types

Brian M. Cox and Charles Chavkin

INTRODUCTION

The initial proposal by Beckett and Casy (1954) that opiate drugs might act through specific receptors to produce analgesic effects was based on a comparison of the analgesic potencies of enantiomeric pairs of opiates and the relative potencies of chemically similar synthetic opiatelike drugs. An extensive study of a complex series of phenylpiperidines led Portoghese (1965) to note that not all the activities observed with these compounds could be explained by a common mechanism of interaction with a single receptor type. He therefore raised the possibility that opiate drugs might be able to interact with more than one type of receptor. Martin and coworkers (Martin 1967; Martin et al. 1976) also found it necessary to postulate the existence of more than one type of receptor in order to account for the differences in the profile of actions exerted by several chemically dissimilar opiates in the chronic spinal dog preparation. Martin et al. (1976) named these receptor types after the prototypic opiate compounds which induced each pattern of actions; morphine acted on μ receptors; ketocyclazocines, on κ receptors; and SKF-10,047 (N-allylnormetazocine), on μ receptors. Despite the different pattern of effects for each of these drugs, Martin regarded the effects of each compound as opiatelike, because all the actions appeared to be antagonized by naloxone or naltrexone (Gilbert and Martin 1976; Martin et al. 1976). These *in vivo* results were supported by results obtained in *in vitro* bioassay preparations. Lord et al. (1977) noted the different relative potencies of selected alkaloid and peptide opioids in inhibiting the electrically stimulated guinea pig ileum (GPI) and mouse vas deferens (MVD) preparations, and differences in their sensitivity to antagonism by naloxone in the same tissues. They were also able to show that the apparent receptor heterogeneity could be confirmed by comparing the relative potencies of several opioids in displacing radiolabelled alkaloid and peptide opioids from binding sites in brain neural membranes. Sites at which the alkaloid opiates interacted in these tissues showed properties similar to those predicted for Martin's μ receptors. Ethylketocyclazocine

appeared to act at a separate site comparable to Martin's κ receptor, while the enkephalins apparently acted at yet another site, clearly distinct from Martin's σ site, which Lord et al. (1977) characterized as δ.

Evidence from numerous in vivo and in vitro studies has documented the existence of μ-, δ-, and κ-type opioid receptors. The existence of additional opioid receptor types has also been proposed, and the properties of ε, μ_1, and σ receptors are under study in several laboratories. ε receptors were first identified by Schulz et al. (1979) and Lemaire et al. (1978) as receptor sites in the rat vas deferens, having high affinity and selectivity for β-endorphin and fragments longer than ß-endorphin (1-19). In this smooth muscle bioassay, the effects of ß-endorphin can be blocked by naloxone with an apparent affinity between 15-27 nM (Gillan et al. 1981; Lemaire et al. 1978). Surprisingly, the benzomorphan opioids, ethylketazocine and bremazocine (potent κ receptor agonists) are pure antagonists in the rat vas deferens (Gillan et al. 1981). ε receptor binding sites in rat brain membranes have been biochemically detected by Chang and coworkers (1984). However, the existence of ε receptors in the rat vas deferens was recently challenged (Smith and Rance 1983).

Very high affinity (K_d<1 nM) binding sites able to bind μ and δ opioids without discrimination were first identified as a subset of μ receptor designated μ_1 by Wolozin and Pasternak (1981). The noncompetitive antagonists naloxazone and naloxonazine, synthesized by Pasternak, are claimed to be selective, irreversible inhibitors of μ_1 binding. μ_1 sites are postulated to be important for mediating opioid analgesia but not respiratory depression (Nishimura et al. 1984; Ling et al. 1985).

The σ receptor first described by Martin (Martin et al. 1976) has also been extensively studied. Whether σ receptors are opioid receptors is a matter of definition and is currently the subject of ongoing debate. The original description of the σ behavioral syndrome included the observation that these effects could be blocked by naltrexone (Martin et al. 1976). In addition, the prototypic ligand N-allylnormetazocine (SKF-10,047) is a derivative of the opioid structure. Yet others have found that SKF-10,047 produces a syndrome in rats and monkeys that is not sensitive to naloxone antagonism (Harris 1980; Brady et al. 1982). Additional studies have shown that the ^{3}H-SKF-10,047 has two different binding sites: one shared by the nonopioid, phencyclidine (Zukin and Zukin 1981; Mendelsohn et al. 1985), and a separate site that binds haloperidol (Su 1982; Tam 1985). Neither site binds naloxone with high affinity. Thus, if we accept the definition that the opioid receptor types must all share the characteristic of sensitivity to the Dpioid antagonists naloxone and naltrexone, then the existence of σ opioid receptors has only been shown in the chronic spinal dog preparation.

The methods described in this review have proven to be quite powerful and will undoubtedly be important for the further

characterization of these additional opioid receptor types. As will be apparent from the following discussion, strategies for the identification of opioid receptor types require converging methodologies, since no single method is unambiguous.

The *in vivo* results of Martin et al. (1976) indicating the presence of more than one type of opioid receptor are now well supported by *in vitro* studies. Methods for the characterization of each receptor type have become available.

The different types of opioid receptors have been shown to differ primarily in their relative affinities for different opioids, although in some cases there is accumulating evidence for differences in the signal transduction mechanisms employed by opioid receptor types (North, this volume). In examining the properties of a receptor mediating a specific effect, it is therefore necessary to examine the affinities and potencies of opioid ligands interacting with that receptor. Opioid receptors, like others, may be characterized by measurement of ligand binding affinities, measurement of agonist potencies in inducing an effect, estimates of agonist affinity constants obtained from measurements of the pharmacological effect, and estimates of antagonist apparent affinity for the receptor by measurements of the potencies of antagonists in inhibiting the actions of opioid agonists. These analyses each provide insight into a different component of agonist action and, when compared for different opioids in different tissues, may allow a profile of the properties of each receptor type to be assembled.

OPIOID LIGAND BINDING: LIGAND DISSOCIATION CONSTANTS (K_d)

The initial interaction of a ligand with its receptor is obviously essential for agonism or antagonism to be observed, and the affinity of a ligand for its receptor is an important determinant of its potency, although pharmacokinetic and other factors may also significantly affect potency. The estimation of K_d therefore provides critical information regarding a ligand's interaction with a receptor. Many studies of radiolabelled opioid ligand interactions with binding sites in membrane preparations from brain and other tissues have now been conducted, following the pioneering studies of Terenius (1973), Pert and Snyder (1973), and Simon et al. (1973). It is now clear that a few opioid ligands bind with high affinity in a manner predicted by a simple Langmuir adsorption isotherm and are thus presumed (in the absence of evidence to the contrary) to bind to a single type of binding site. An example of such a ligand is [^{3}H-D-Ala^2-MePhe^4-Gly-ol^5]enkephalin (DAGO), which appears to have high selective affinity for μ-type opioid receptors (Gillan and Kosterlitz 1982). However, most opioids show a more complex interaction with neural membrane preparations. By suitable curve fitting procedures, the plot of amount of specifically bound ligand vs. ligand concentration can usually be resolved into a summation of two or more Langmuir adsorption isotherms (Munson and Rodbard 1980). The use of such procedures, especially when comparisons are made between the

binding affinities of both selective and nonselective opioids in displacing a series of selective radiolabelled opioids. enables the apparent affinities of many ligands at several types of binding sites to be determined (Werling et al. 1985).

A question arises as to the significance of binding affinities measured in this way. Most studies of this kind have employed buffer media lacking ions critical for tissue function, at temperatures lower than that at which the tissue usually functions. Such abnormal conditions tend to increase the ratio of specific to nonspecific binding of opioid radioligands, thus making quantitation of binding more reliable and possibly increasing the stability of the ligands used. Opioid agonist binding is generally reduced in the presence of sodium and is also sensitive to changes in incubation temperatures (Simantov et al. 1976). It is uncertain to what extent variations in environmental conditions affecting agonist affinity also affect receptor selectivity. However, it appears that the discrimination of opioid receptor types observed under optimum binding conditions can still be observed when incubations are conducted in a modified Krebs medium at 37 °C (Werling et al. 1985; Zarr et al. 1986). A comparison of ligand selectivities measured in Tris buffer and modified Krebs is presented in table 1. The selectivity of the agonists is generally not impaired, although [D-Ser^2-Leu^5]enkephalyl-Thr (OSLET)

TABLE 1. *Comparison of opioid agonist receptor selectivities determined by displacement of radiolabelled ligands in Tris buffer or modified Krebs buffer*

		Receptor Types		
Ligand	Conditions (°C)	μ	σ	κ
DAGO	Tris, 25°*	[8.36]	130	170
	Krebs, 37°**	[8.34]	330	>200
DSLET	Tris, 25°	20	[8.74]	>1,000
	Krebs, 37°	9	[8.05]	>110
U50488H	Tris, 25°	1,300	12,000	[9.14]
	Krebs, 37°	>280	>280	[8.46]

*Data from James and Goldstein (1984) using guinea pig brain membranes.

**Data computed from Werling et al. (1985) using guinea pig cortex membranes.

NOTE: Binding selectivity profiles are tabulated according to the convention proposed by James and Goldstein (1984). Figures In square brackets are the negative logarithms of the K_1 values (in molar units) for each displacing ligand at the site at which thls ligand has highest affinity (the preferred site). Other values are ratios of the K_i at the designated site to the K_1 at the preferred site. Values preceded by the > symbol are minimal values; the K_1 at the nonpreferred site exceeds 1 μM and is not accurately determined.

(Gacel et al. 1980) appeared to show less discrimination between μ and μ binding sites in the modified Krebs medium. On the basis of such studies, ligands with good selectivity for each type of opioid receptor can be established, and the ligand binding properties of each type of receptor can be defined in terms of their relative affinities for a few select ligands; μ receptors bind DAGO with much higher affinity than DSLET, [D-Pen^2-D-Pen^5]enkephalin (DPDPE) (Mosberg et al. 1983). or U50488H (Lahti et al. 1982); δ receptors bind DPDPE or DSLET with much higher affinity than DAGO or U50488H; and κ receptors bind U50488H with much higher affinity than DAGO, DSLET, or DPDPE. This type of relative affinity comparison has become very useful in the functional characterization of opioid receptor types (Gillan and Kosterlitz 1982; James and Goldstein 1984; Werling et al. 1985).

AGONIST POTENCY ESTIMATES (A_{50} AND K_a)

Agonist-dependent and tissue-dependent factors that determine the magnitude of a tissue's response to an agonist are summarized in table 2. Differences in the ability of drugs to gain access to receptors and differences in their stability in tissues are often neglected as possible contributors to relative potency differences

TABLE 2. *Components of agonist action*

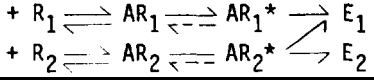
Agonist dependent

1. Access to receptor; ligand stability
2. Affinity (K_d)
3. Intrinsic efficacy (ε)

Tissue dependent

4. Receptor concentration ([R_o]); spare receptors
5. Receptor heterogeneity (R_{o1}, R_{o2}, etc.)
6. Stimulus-effect relationship (E/S)

Model



NOTE: In the model, A represents an agonist; R_1, R_2 represent different receptors interacting with A; AR_1* and AR_2 are activated forms of each agonist receptor complex (e.g., following an agonist-induced conformational change and/or an interaction with another macromolecule); and E_1, E_2 are effects produced as a result of receptor activation by agonist A.

between tissues, but clearly these differences require consideration. The demonstration that peptidase inhibitors significantly increase the apparent potencies of some but not all opioid

peptides in bioassay preparations (Corbett et al. 1984; James and Goldstein 1984) emphasizes the importance of demonstrating agonist stability in quantitative estimates of potencies, if artifactually low estimates of potency are to be avoided.

If completely specific agonists or antagonists for each type of opioid receptor were available, then determination of the type of opioid receptor mediating a particular effect could be made simply by observing in a qualitative fashion whether or not a response was induced (or antagonized) by each type of specific agent. Since absolute specificity is at present not attained in opioid receptor ligands, some consideration must be given to determining the degree of selectivity that is required for unambiguous receptor identification by qualitative demonstration of activity induced by a partially selective ligand. Unfortunately, the required level of selectivity for agonists is very high; substantial differences in agonist efficacies and tissue receptor concentrations will greatly affect the potency of an agonist and thus reduce the reliability of potency estimates in qualitative discrimination of receptor types. The situation is less critical for antagonists, where the specificity is determined exclusively by its affinity for each receptor type. Here, information from radioligand binding studies is relevant; it is readily apparent from radioligand displacement studies that very clear discrimination of receptor types can be achieved with ligands with more than about 200-fold selectivity for a particular receptor type. Competitive antagonists for specific opioid receptor types with this degree of selectivity are not yet available, although the σ-selective peptide antagonist N,N-diallyl-Tyr-Aib-Aib-Phe-Leu-OH (ICI 174,864) approaches this level of selectivity (Corbett et al. 1984). It is therefore necessary to make quantitative estimates of agonist potencies and from these to compute functional agonist affinities.

A convenient measure of agonist potency is the concentration of agonist required to produce 50 percent of maximum effect in a particular preparation (A_{50}). This value can be obtained by interpolation in concentration-response curves or by computation from a theoretical curve fitted to the experimental data (Black and Leff 1983). However, A estimates alone are not reliable indicators of agonist affinity for the receptors mediating the observed effect. In addition to binding affinity, observed agonist potency is often controlled by the efficacy of the agonist, receptor reserve in the tissue, and the ability of the intact drug to penetrate to the site of action. These are characteristics that greatly differ among the opioids available and limit the value of simple potency estimates in characterizing receptors.

Furchgott (Furchgott and Bursztyn 1967; Furchgott 1978) has proposed a method by which functional estimates of agonist affinity can be obtained from comparisons of agonist dose-response curves constructed before and after irreversible inactivation of a fraction of the receptors. This is essentially a null-point method in

which equal effects are assumed to result from an equal stimulus to the effector system; in the model outlined in table 2, for example, an equal number of activated agonist-receptor complexes. The second assumption is that the noncompetitive antagonist used does not alter the binding affinity of the residual receptor sites or the efficacy of the agonist. The development of the irreversible opioid antagonists ß-chlornaltrexamine (ß-CNA) and ß-funaltrexamine (ß-FNA) (Portoghese et al. 1979; Portoghese et al. 1980) has enabled this method to be applied to the computation of opioid agonist affinities. Figure 1 shows dynorphin A(1-13) dose-response curves in guinea pig ileum and mouse vas deferens preparations, before and after ß-CNA treatment. It is apparent that the receptor reserve for the receptors through which dynorphin exerts its inhibitory effect is greater in guinea pig ileum than in mouse deferens, since the same ß-CNA treatment produced a greater reduction in maximum effect in vas than in ileum. However, when reciprocals of doses producing equal effects before and after ß-CNA treatment are plotted and the K_a values computed from the slopes and intercepts, it appears that dynorphin had a very similar affinity for the receptors mediating its effects in each tissue. Other evidence also points to this conclusion (Cox and Chavkin 1983), suggesting that despite considerable differences in potency, dynorphin acts in both mouse vas deferens and guinea pig ileum through κ-type opioid receptors. Opioid agonist affinities have been estimated in other systems by the same technique; some representative values from the literature are listed in table 3. In each case, the affinity estimates are higher than the A_{50} values obtained in the same preparation. The ratio of K_a to A_{50} gives an approximate indication of the magnitude of the receptor reserve in each tissue.

Some indication of the magnitude of the receptor reserve in a bioassay preparation can also be obtained by examination of the effect of treatment with an irreversible antagonist on the agonist log. concentration-response curve. An example of such an experiment is shown in figure 2. Normorphine, dynorphin A(1-13), and [Leu^5]enkephalin act through μ, κ, and δ opioid receptors, respectively, to inhibit contractions of mouse vas deferens preparations. After ß-CNA treatment, the maximum response obtainable with normorphine and dynorphin A(1-13) was considerably reduced. However, the log. concentration-response curve for [Leu^5]enkephalin was shifted to the right, with no reduction in maximum response after the same ß-CNA treatment. A higher concentration of ß-CNA produced a further parallel shift to the right in the [Leu]enkephalin curve. The simplest explanation of these results is that after destruction of a fraction of the μ, κ, and δ receptors by covalent occupation by ß-CNA, insufficient μ and κ receptors were left for agonists acting through these receptors to produce full inhibition. However, sufficient δ receptors remained for a full inhibitory response to be obtained. Thus, there was a greater reserve of δ receptors than μ or κ receptors in this tissue.

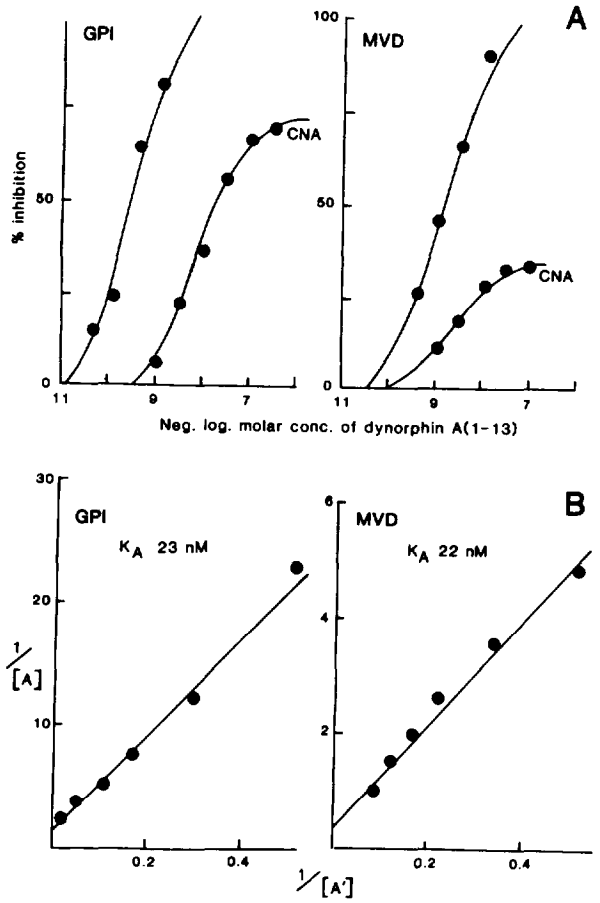


FIGURE 1. *Estimation of dynorphin affinity at κ receptors in guinea pig ileum and mouse vas deferens*

NOTE: (A) Log. concentration-response curves for dynorphln A(1-13) on the inhibition of stimulated responses In isolated guinea pig ileum (GPI) and mouse vas deferens (MVD), before and after exposure to ß-CNA (3 nM, 20 minutes, followed by 90 minutes of repeated washing). Each data point represents a mean value from estimates obtained in four preparations (for methods, see Chavkln and Cox (1983)). (B) Furchgott plots, showing reciprocals of equieffective concentrations of dynorphln A(1-13) before and after ß-CNA treatment, are shown. The K_a values for dynorphin A(1-13) In each tissue were computed from the equation K_a [(slope-1)/intercept] (Furchgott 1978). Data from Chavkin and Cox (unpublished).

For all three types of opioid receptors considered here, there appears to be a substantial receptor reserve in the tissues listed in table 3. However, it should be noted that the estimates of

TABLE 3. *Estimates of agonist affinity*

Ligand		K_a (μM)	A_{50} (μM)	Ratio (K_a/A_{50})
μ Receptors				
Normorphine[a]	GPI	1.5	0.25	6
Normorphine[b]	Rat LC slice	12	1.2	10
Morphine[c]	Rat hot plate	1.7	0.2	8
[Met^5]enkephalin[b]	Rat LC slice	16	0.6	25
OADLE[b]	Rat LC slice	2	0.15	13
δ Receptors				
[Leu^5]enkephalin[d]	MVO	2	0.02	100
κ Receptors				
Dynorphin A[d]	MVO	0.02	0.0014	16
Dynorphin A[d]	GPI	0.02	0.0003	88

KEY: GPI, guinea pig ileum; CC, locus coeruleus; MVD, mouse vas deferens.

[a]Porreca and Burks (1983).

[b]Williams and North (1984).

[c]Tallarida and Cowan (1982).

[d]Cox and Chavkin (unpublished).

functional agonist affinity are most readily made in tissues where agonist potency is initially very high; that is, where there is very probably a substantial receptor reserve. No conclusions about the generality of the occurrence of spare receptors in opioid receptor populations can therefore be made from this very limited sampling of tissues. Recent studies have demonstrated that opioid tolerance is associated with a reduction in opioid receptor reserve, at least in guinea pig ileum myenteric plexus (Chavkin and Goldstein 1984).

As further studies are completed, it seems probable that it will be possible to characterize the various types of opioid receptors by examination of the agonist affinity constants for a few relatively selective agonists. It should be noted that the Furchgott method depends on a double-reciprocal plot. Alternative methods in which nonlinear curve-fitting procedures are used to fit the experimental data have been proposed (Parker and Waud 1971; Black et al. 1985). The errors inherent in the estimates of opioid

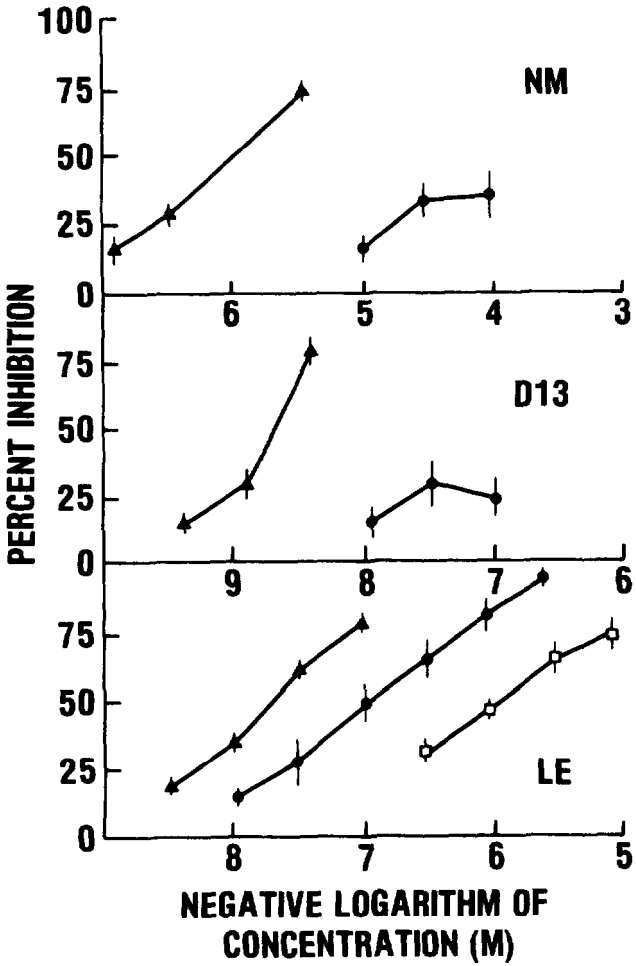


FIGURE 2. *Effects of ß-CNA treatments on log. concentration-response curves for normorphine (NM; upper panel), dynorphin A(1-13) (D13; middle panel), and [Leu^5]enkephalin (LE; lower panel) in isolated mouse vas deferens*

KEY: ▲ = prior to treatment; ● = after ß-CNA. 1 nM for 20 minutes; □ = after ß-CNA, 3 nM for 20 minutes.

NOTE: After pretreatment, the tissues were washed repeatedly for 90 minutes to remove noncovalently bound ß-CNA before the log. concentration curves were redetermined. Results are the mean values from tour preparations; vertical bars are estimates of the standard errors of the mean values. The ß-CNA 1-nM treatment clearly reduced the maximum response to normorphine and dynorphin A(1-13), while producing a parallel shift to the right In the [Leu^5]enkephalin log. concentration-response curve. (For details of methods, see Cox and Chavkin (1983)).

agonist K_a have generally not been calculated, but may be significant in relation to affinity estimates which differ by less than one or two orders of magnitude. For this reason, receptor discrimination will be most reliably achieved if receptor affinities are estimated with agonists with considerable selectivity for each receptor type.

ESTIMATES OF ANTAGONIST AFFINITY CONSTANTS (K_e)

Classically, estimation of antagonist affinity constants has been the method of choice in receptor discrimination (Schild 1947). Kosterlitz and coworkers (Kosterlitz and Watt 1968; Lord et al. 1977) have shown the utility of estimates of naloxone affinity in characterization of opioid receptors. However, naloxone has the disadvantage that its affinities for δ- and κ·type opioid receptors are very similar, and only about tenfold lower than its affinity for μ receptors. Thus, estimates of naloxone K_e have been valuable in discriminating receptors mediating opioid effects in tissues where the functional contribution to the actions of an agonist of either δ or κ receptors can be shown to be negligible, on the basis of independent evidence (e.g., in guinea pig ileum (Lord et al. 1977) or in NG 108-15 cells (Sharma et al. 1975)). However, naloxone cannot reliably discriminate between δ and κ receptor-mediated effects in mouse vas deferens preparations (Cox and Chavkin 1983). There is generally good agreement between estimates of naloxone affinity for opioid receptors based on displacement of the specific binding of radiolabelled agonists and on antagonism of their pharmacological effects (table 4).

There are few other pure antagonists of opioid actions. Of the currently available compounds, diprenorphine, a partial agonist with weak agonist effects in some opioid systems, comes closest to being a universal antagonist at all types of opioid receptors. This attribute makes it useful as a radiolabelled ligand in displacement studies examining selective agonists but limits its value in the direct discrimination of receptor types. A recently developed enkephalin analogue (ICI 174,864) has very good selectivity for δ receptors (Cotton et al. 1985) but relatively low potency (table 4). Further studies are needed to determine how useful this compound will be in pharmacological discrimination of receptor types. A selective antagonist for κ receptors is not yet available. The benzomorphan compound Mr 2266 has comparable antagonist potencies at μ, δ, and κ receptors (Lord et al. 1977) but also has weak agonist actions in some systems.

The use of partially selective antagonists may give ambiguous results in receptor classification if nonselective agonists are used in an experimental system where the same effect can be induced by more than one receptor type. An example is provided by studies of the actions of opioid peptides derived from prodynorphin on isolated guinea pig ileum preparations (Chavkin and Goldstein 1981; James et al. 1984). In these studies, naloxone gave K_e values of 3 to 4 nM against the normorphine standard, a fairly selective μ agonist. K_e values of about 30 to 40 nM were observed against

TABLE 4. *Estimates of opioid antagonist affinity constants*

Receptor Type/ Antagonists	Procedures	Affinity Constant, nM
μ Receptors		
Naloxone	Displacement of ^{3}H-DAGO, brain	2[e]
	Guinea pig ileum, antagonism of DADLE	2[e]
	Guinea pig ileum, antagonism of normorphine	1[a,e]
	Mouse vas, antagonism of normorphine	4[c,d]
	Rat locus coeruleus, antagonism of normorphine	3[f]
	Rat locus coeruleus, antagonism of DADLE	3[f]
κ Receptors		
Naloxone	Displacement of ^{3}H-EKC, brain (μ block)	17[e]
	Guinea pig ileum, antagonism of EKC	23[a]
	Guinea pig ileum, antagonism of dynorphin A	91[a]
	Mouse vas, antagonism of dynorphin A	18[d]
δ Receptors		
Naloxone	Displacement of ^{3}H-DADLE, brain (μ block)	30[b]
	Displacement of ^{3}H-DPDPE, brain	23[b]
	Mouse vas, antagonism of [Leu^{5}]enkephalin	24[c]
ICI 174,864	Displacement of ^{3}H-DADLE, brain (μ block)	170[b]
	Displacement of ^{3}H-DPDPE, brain	190[b]

[a]Chavkin et al. (1982).

[b]Cotton at al. (1985).

[c]Cox and Chavkin (1983).

[d]Gillan and Kosterlitz (1982).

[e]Magnan at al. (1982).

[f]Williams and North (1984).

dynorphin A and dynorphin B, which are thought to act preferentially with κ receptors. However, peptides of intermediate length such as dynorphin A(1-8), a-neo-endorphin, and ß-neo-endorphin gave intermediate naloxone K_e values ranging from 8 to 22 nM. The simplest interpretation of these intermediate values is that these agonists exert effects on the release of acetylcholine in guinea pig ileum by interactions with both μ and κ receptors. The intermediate K_e values may arise as a weighted average K_e determined by the relative contributions of each receptor type to the agonist effect of the peptide. Thus, the antagonist K_e alone is not a useful guide to the receptors mediating a response unless it is determined against a fairly selective agonist. In estimates of naloxone K_e, it is desirable to construct agonist concentration-response curves at several concentrations of antagonist and to determine the slope of the Schild plot (log (DR-1) v.log. antagonist concentration (Schild 1947)). The Schild equation assumes that only a single receptor binding site is involved; therefore, slope values significantly different from 1.0 will invalidate the estimate of agonist affinity.

Quantitation of antagonist potencies after in vivo administration can also be used in the characterization of receptors. Competitive opioid antagonists produce a parallel shift to the right of in vivo log. administered dose-response curves that is analogous to the-shift of in vitro log. concentration-response curves (Cox and Weinstock 1964; Smits and Takemori 1970). Pseudo-Schild plots can be constructed and in vivo apparent pA_2 values computed. However, it should be noted that these values are not quantitatively identical to pA_2 estimates obtained from in vitro concentration-effect curves. This estimate is related to the true antagonist affinity constant by proportionality factors (usually unknown) which reflect the relationship between administered dose and the free drug concentrations of both agonist and antagonist in equilibrium with the receptor (Tallarida et al. 1979). Since proportionality between dose and tissue concentration may not hold over a large range of agonist or antagonist concentrations, and the time of peak tissue concentration of drug (which is most likely to be directly proportional to administered dose) may differ for agonist and antagonist, receptor discrimination by estimates of in vivo pA_2 is potentially subject to additional sources of error which limit its usefulness.

Irreversible antagonists which preferentially interact with one class of receptors can also be used to identify receptor types mediating a pharmacological effect. The naltrexone analogue ß-FNA appears to act irreversibly with μ receptors (Portoghese et al. 1980). Thus, actions mediated exclusively through μ receptors can be antagonized by ß-FNA. It is important in the use of irreversible antagonists to confirm that the observed antagonism is irreversible. This is necessary, because such agents may also display reversible components of action which do not demonstrate the receptor selectivity of the irreversible component. ß-FNA shows κ receptor agonist activity which may dominate the profile of actions observed shortly after ß-FNA administration (Ward et al. 1982). However, this action is reversible; at longer time periods,

a selective antagonism of opioid action at μ receptors is observed. The irreversible nature of this antagonistic effect can be demonstrated by showing inability to overcome the antagonism by the addition of higher doses of μ agonist (Ward et al. 1982; Gmerek and Woods 1985). Another irreversible antagonist with selectivity for μ receptors is naloxonazine (Hahn et al. 1982). Unlike ß-FNA, however, this agent appears to have selective affinity for a subset of μ receptors, designated by Wolozin and Pasternak (1981) as μ_1. Irreversible antagonists which can act selectively with δ or κ receptors are not currently available; their development would considerably aid the characterization of opioid receptor types.

When used in vivo, irreversible receptor ligands have a duration of effect which considerably exceeds the duration of action of naloxone or naltrexone. However, their duration of action is not indefinite; antagonized responses usually return 3 or 4 days after treatment (Portoghese et al. 1979). This is generally assumed to result from the synthesis and integration into neural membranes of newly synthesized receptors which are not occupied by the irreversible antagonist.

Quantitative characterization of opioid receptor types is now feasible by several methods which examine different aspects of ligand interaction with receptor and the induction of agonist effect. Application of these approaches to opioid receptor classification, using receptor-type selective agonists and antagonists, should lead to the development of profiles of the properties of each type of receptor which will be useful in defining the receptors mediating opioid action in other systems.

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Dynorphin: Specific Binding to Opiate Receptors

Nancy M. Lee and Herbert Landahl

INTRODUCTION

The fact that multiple receptors and multiple endogenous ligands exist in the opioid system is now well established. The receptors include μ, selective for morphine and related compounds; δ, selective for enkephalins; κ, selective for ethylketocyclazocine, and probably others as well (Martin et al. 1976; Lord et al. 1977). The endogenous opioid peptides can be grouped into three major classes with members of each sharing a common precursor: ß-endorphin and related peptides, the enkephalins, and dynorphin and α-neo-endorphin (Rossier 1982).

Since one assumes that every endogenous ligand must interact with a specific receptor, it is tempting to hypothesize that each of the known receptor types interacts with a different class of endogenous opioids. In the case of the δ receptor and the enkephalins, the correlation is clear, but in the case of the μ and κ receptors, the picture is not so simple. ß-endorphin is known to interact with the μ receptor, but it binds equally well to δ receptors (Lord et al. 1977). Furthermore, it has not been established whether still another so-called ξ receptor is responsible for some of its physiological effects (Wood 1982).

With regard to the κ receptor, it has been suggested that dynorphin is the endogenous ligand, because it behaves like the κ drug ethylketocyclazocine in the guinea pig ileum (Huidobro-Toro et al. 1981), and because it binds to κ sites in mammalian brain (Chavkin et al. 1982). However, this peptide has many in vivo effects that are quite Unlike those of typical κ agonists. Although not analgesic itself, it antagonizes morphine- or ß-endorphin-induced analgesia in naive mice and, even more remarkably, potentiates the analgesic effects of these opioids in tolerant animals (Tulunay et al. 1981); this potentiation is also seen in the chronically treated guinea pig ileum (Rezvani and Way 1984). Dynorphin also potentiates morphine-induced respiratory depression, again while having no effect by itself (Woo et al. 1983).

These findings suggest that while dynorphin may interact with κ-receptors in the brain, it probably interacts with other types of opioid receptors as well. In order to explore this question, we have examined the binding of dynorphin to brain homogenates and its effect on the binding of other opioid ligands.

DYNORPHIN COMPETITION WITH OTHER LIGANDS

The ability of dynorphin to interact directly or indirectly with different types of opiate receptors was first tested by determining the effect of this peptide on the in vitro binding of various tritiated opioid ligands to a brain membrane fraction. As shown in table 1, dynorphin is able to compete with a number of different opiates, including dihydromorphine (DHM), a prototypical μ ligand; D-Ala^2-D-Leu^5 enkephalin (DADLE), a δ drug, and ethylketocyclazocine (EKC), a κ agonist. In further studies, a range of concentrations of the unlabelled drug was used and dynorphin's effect on the resulting Scatchard plots determined. The binding was analyzed on the assumption that there were two populations of binding sites for each ligand; in every case, dynorphin's main effect was to reduce B_{Max} of the high-affinity site in a dose-related manner. In the case of EKC, dynorphin also increased the high-affinity K_d (Garzon et al. 1984). These results establish that dynorphin interacts with not only receptors for κ ligands. but for the others as well.

TABLE 1. *The potency of dynorphin 1-13 to inhibit binding of various opiate ligand binding to mouse brain P_2 fraction*

Tracer Drugs	Apparent IC_{so} (nM)
Dihydromorphine	10
Etorp ine	160
D-Ala^2-D-Leu^5-enkephalin	50
D-Ala^2-Met^5-enkephalinamide	14
Ethylketocyclazocine	17
Naloxone	52

NOTE: The concentration of radiolabelled ligand used was 0.5 nM. The ligand and dynorphin$_{1-13}$ were added simultaneously.

DYNORPHIN INTERACTION WITH SPECIFIC OPIOID SITES

While the studies just discussed suggest possible heterogeneity in dynorphin binding sites, it is difficult to conclude anything about the specific sites involved, because each of the displaced ligands may interact with several sites. Thus DADLE has been reported to interact with μ as well as δ sites (Chang and Cuatrecasas 1979), while EKC binds to μ and δ in addition to κ

sites (Pfeiffer and Herz 1981). A further problem with Scatchard plots is that it is difficult'to cover a wide range of drug concentrations, because data at very low and high concentrations (i.e., binding to highest and lowest affinity sites) are subject to large experimental error. Error also is increased because both ordinates depend on the free drug concentration, which may be difficult to determine with precision.

In order to gain insight into dynorphin's interaction with specific sites, we used a competition technique, coupled with computer analysis. To define the sites involved, we initially studied the effects of tritiated dihydromorphine (DHM), D-Ala^2-D-Leu^5-enkephalin (DADLE), and ethylketocyclazocine (EKC) on the binding of the corresponding unlabelled ligands. The resulting data generated nine competition curves (figure 1). These could then be compared with theoretical curves generated by a computer program which assumed various numbers of binding sites and various values for different parameters associated with-these sites.

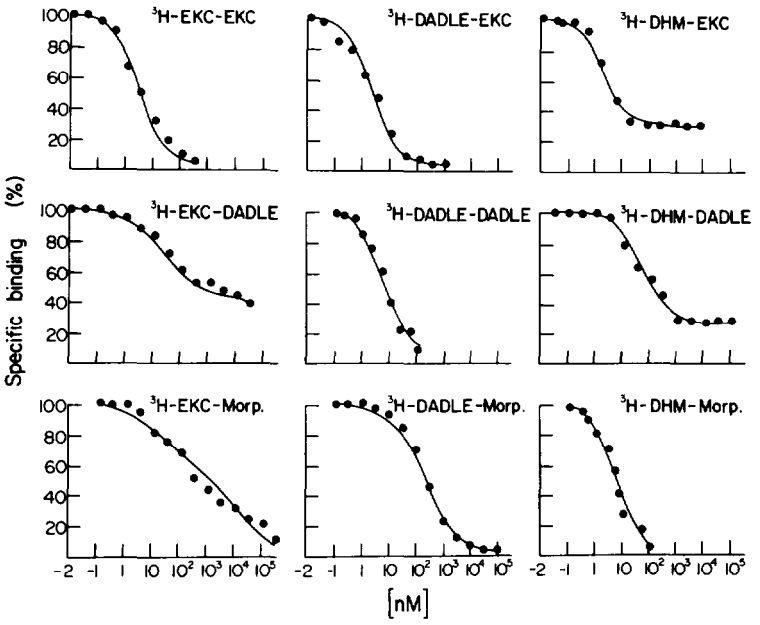

FIGURE 1. *Competition curves*

NOTE: Binding of tritiated EKC, DADLE, and DHM was assayed in the presence of various concentrations of each of these unlabelled ligands (substituting morphine for DHM). Theoretical curves are Indicated by solid lines.

To analyze the binding data, we used the following mathematical model. For each site i, of total number R_{oi}, the number of tracer molecules of concentration (T) bound to the receptor will be $(TR_i) = (T)(R_i)/K_{iT}$, where R_i is the total number of occupied receptors and K_{iT} is the dissociation constant of receptor i for tracer T; likewise, the number of drug molecules of concentration D bound to the receptor will be:

$$(DR_i) = (D)(R_i/K_{iD}).$$

The total number of R_{oi} is just the sum of the free R_i plus (TR_i) and (DR_i) from which:

$$R_i = R_{oi}/(1 + T/K_{iT} = D/K_{iD}) \qquad (1)$$

so that

$$(TR_i) = R_{oi}T/(K_{iT} + T + DK_{iT}/K_{iD}). \qquad (2)$$

The measured value YTD is the ratio of the sum of the amounts of tracer bound to each receptor in the presence of tracer and drug (in amount D) divided by the total amount bound when the tracer has a concentration T, but D equals 0. That is, the specific binding for each of the nine curves in figure 1 is given by:

$$Y_{TD} = \Sigma(TR_i)/\ \Sigma(TR_i)_{D=0} \qquad (3)$$

or substituting (2) into (3).

$$Y_{TD} = \frac{\Sigma(R_{oi})/(K_{iT} + T + DK_{iT}/K_{iD})}{\Sigma R_{oi}/(K_{iT} + T)} \qquad (4)$$

If T and D refer to the same drug, T and D are concentrations of labelled and unlabelled drug, respectively, and K_{iT} equals K_{iD}. In all experiments, T equals 0.5 nM. The quantity T is retained in equation (4), since a number of K values were found which were substantially less than T.

The best fit under these conditions for the data shown in figure 1 proved to be a model assuming four independent sites. In this model, site 1 was most selective for DADLE, sites 3 and 4 were most selective for DHM, and site 2 bound only EKC, although the latter drug also interacted with sites 1 and 3.

We next applied this technique to dynorphin 1-17 and some related peptides. The latter included dynorphin 1-5 through 1-13, α- and λ-neo-endorphin, and dynorphin B. An inspection of the competition curves using these ligands with ^{3}H-DHM showed that most of these compounds competed to some extent with DHM at site 3. These affinities are shown in table 2. A similar approach was applied to the isplacement of ^{3}H-DADLE, to obtain K values for site 1, and to ^{3}H-EKC, for K values for site 2 (table 2).

TABLE 2. *Dissociation constants for dynorphin fragments (mM)*

Competing Drugs	Sites #1	#2	#3
Dyn 1-5	4.1	1,500.0	63.00
1-6	113.0	2.0	342.00
1-7	126.0	4.0	355.00
1-8	106.0	10.0	1,735.00
1-9	20.0	13.0	1.60
1-10	24.0	6.0	5.50
1-11	8.1	2.0	0.24
1-12	43.0	10.0	0.89
1-13	14.0	13.0	1.20
1-17	10.0	25.0	0.75
λ-neo-end	46.0	0.4	30.00
Dyn B	36.0	100.0	1.40
DHM	252.0	19,860.0	4.70
DADLE	4.0	56,700.0	50.80
EKC	1.7	2.3	3.50

In conclusion, our binding data indicate that dynorphin-related peptides bind to all three major sites in our analysis. Since enkephalins have the greatest affinity for site 1, and morphine has the greatest affinity for site 3, these sites correspond to the μ and δ classes of conventional terminology. Furthermore, as the peptide becomes longer, the affinity for site 3 generally becomes higher relative to that of site 1.

KINETICS OF DYNORPHIN INHIBITION OF OPIATE BINDING

The time course of the inhibitory effect of dynorphin 1-13 and 1-17 on tritiated naloxone binding at 37 °C is presented in tables 3 and 4. Peptides were added in a concentration of 100 nM 15 minutes after naloxone (the antagonist at this temperature requires less than 4 minutes to reach equilibrium), and specific binding of ^{3}H-naloxone was monitored. The maximum inhibition was obtained during the first 5 minutes and was practically identical for both peptides. After this period, the inhibition decreased very slowly, the half-time or recovery of naloxone binding being approximately 40 minutes for dynorphin 1-13 and 90 minutes for dynorphin 1-17. In a parallel study, the remaining free form of the peptide was evaluated over these time intervals using the mouse vas deferens (MVD) bioassay.

In further studies, we unexpectedly found that dynorphin inhibits *in vitro* binding even if the peptide is administered *in vivo* and

TABLE 3. *Time course of the inhibitory effect of dynorphin 1-13 on ^{3}H-naloxone (1 nM) specific binding*

Time After Dynorphin 1-13 (100 nM) (Minutes)	Specific ^{3}H-Naloxone Binding* (Percent)	Detectable Peptide (MVD)** (Percent)
5	17	18
10	23	16
15	27	5
20	28	-
30	44	-
45	60	-
60	90	-
75	92	-
90	108	-

*Tritiated naloxone (1 nM) was equilibrated (15 minutes at 37 °C), then peptide was added to a final concentration of 100 nM. Binding was determined and specific binding expressed as percentage of the controls which received buffer Instead of the peptlde.

**Samples received peptlde without ^{3}H-naloxone. The amount of peptide present was calculated from a dose response curve and then expressed as percent of the initial concentration added.

TABLE 4. *Time course of the inhibitory effect of dynorphin 1-17 on ^{3}H-naloxone (1 nM) specific binding*

Time After Dynorphin 1-17 (100 nM) (Minutes)	Specific ^{3}H-Naloxone Binding (Percent)	Detectable Peptide (MVD) (Percent)
5	10	70
10	17	50
15	22	30
20	18	20
30	20	10
45	35	5
60	40	-
75	47	-
90	50	-
105	70	-
120	82	-
150	90	-
180	98	-

NOTE: The experimental procedure Is identical to that described In table 3, except that dynorphin 1-17 instead of 1-13 was studied.

washed away during preparation of the brain membranes. In these experiments, mice were injected intracerebroventricularly (ICV) with various doses of dynorphin and sacrificed after various periods of time for tissue preparation. Significant inhibition of *in vitro* binding of both ^{3}H-DHM and ^{3}H-DADLE was observed following an ICV dose of dynorphin as low as 1 µg (Garzon et al. 1984). This effect was observed with animals sacrificed up to 6 hours after this dose, while binding returned to normal after 12 hours. This inhibition was not affected by subjecting the brain membranes to extensive washing, suggesting that it was not due to the presence of free dynorphin in the tissue.

Further studies of this novel finding showed that shorter fragments of dynorphin did not have this effect (Garzon et al. 1984), indicating that the inhibition was probably not due to a dynorphin metabolite. However, inhibition could be reversed by incubation of the membranes from dynorphin-treated mice with either morphine or with Na^{+} ion (Garzon et al. 1984). Our interpretation of this is that the inhibition may be due to a longlasting association of dynorphin with its receptor(s), with dynorphin being displaced by morphine or Na^{+}. This conclusion is consistent with the observation that dynorphin dissociates very slowly from its receptors and suggests that it may be the endogenous ligand reported by others to interfere with *in vitro* opiate binding assays (Simantov et al. 1976).

CONCLUSIONS

Our initial studies of the *in vivo* action of dynorphin indicated that it was unique among opiates. Although having no analgesic properties of its own, dynorphin was able to modulate the analgesia induced by morphine or ß-endorphin. Furthermore, the direction of this modulation--enhancement or inhibition--depended on the state of the animal.

The studies discussed here further support the notion of the uniqueness of dynorphin and provide some clues to the mechanisms underlying its modulatory action. Thus, although we have found that dynorphin interacts with κ type opioid sites, as others have reported, we also have evidence that it interacts with µ and δ sites as well. This interaction with multiple binding sites in itself is not unusual, as other endogenous opioids, such as ß-endorphin and the enkephalins, also bind to more than one site. However, the broad range of its interactions, together with the apparently complex manner in which it interacts with these various sites, suggests that dynorphin may have special modulatory properties. It is also possible that some of the effects of dynorphin are exerted through as yet unidentified receptors, that is, ones that are not labelled by even such a nonspecific opioid agonist as EKC.

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Selective Radioligands for Characterization and Neuroanatomical Distribution Studies of Brain Opioid Receptors

R. Suzanne Zukin, Ann Tempel, and Mahboubeh Eghbali

INTRODUCTION

Opiates and opioid peptides produce actions on the nervous system by interaction with μ, δ, and κ opioid receptors and related σ receptors. These receptors exhibit differing ligand selectivity patterns, diverse neuroanatomical distribution patterns, differing physiological and behavioral profiles, and differing sensitivities of opioid agonists at each site to naloxone antagonism (Snyder 1984; Zukin and Zukin 1984). Most of the classical opiate drugs and endogenous opioid peptides interact with more than one of these receptor sites. Thus, the complex neuropharmacological actions of a given opioid would appear to reflect interaction with varying potencies at a combination of these sites.

Opioid receptors are membrane associated, and their integrity appears dependent upon protein (Pasternak and Snyder 1974), carbohydrate (Howells et al. 1982), and phospholipid (Lin and Simon 1978) components. Studies in neuronal cell lines demonstrating opioid-induced inhibition of adenylcyclase (Klee and Nirenberg 1976; Sharma et al. 1975), as well as studies in brain and cell lines showing guanyl nucleotide modulation of agonist binding (Blume 1978; Childers and Snyder 1978; Zukin et al. 1980), suggest that μ and δ receptors are coupled to cyclase through an inhibitory guanyl nucleotide binding protein (N_i). The molecular dimensions of the receptors have been estimated in prelabelling experiments and, more directly, in partially purified preparations. Experiments in which opioid receptors were prelabelled with the potent agonist [^{3}H]etorphine (Simon et al. 1975) or with u-selective radiolabelled enkephalin derivatives (Childers and Snyder 1978) prior to solubilization afforded a molecular weight estimate of 300 to 400,000 daltons. More recently, solubilization studies of active receptor species from rat brain (Howells et al. 1982; Bidlack et al. 1981; Chow and Zukin 1983), toad brain (Ruegg et al. 1981), and cell cultures (Simonds et al. 1980) have provided similar molecular weight estimates and suggest that the receptors are aggregates of multiple protein components.

The μ, δ, κ, and σ receptors can be distinguished by their ligand selectivity patterns (table 1). The μ receptor exhibits highest affinity for morphine and related morphinelike alkaloids (normorphine, dihydromorphine, and levorphanol), as well as for certain synthetic enkephalins which have bee modified to confer μ-like character. The latter include D-Ala^2, N-Phe^4-Met(O)ol^5-enkephalin (FK 33824) (Romer et al. 1977), which exhibits somewhat higher affinity for μ than for δ receptors (Kream and Zukin 1979), and D-Ala , N-Me-Phe , Gly-ol^5-enkephalin (DAGO) (Handa et al. 1981), which exhibits a 220:1 preference for μ:δ receptors. The δ receptor exhibits preferential affinity for the naturally occurring Met-^{5} and Leu-^{5} enkephalins and certain of their congeners. A relatively high selectivity for the δ receptor is exhibited by D-Ala , D-Leu^5-enkephalin (DADLE) and D-Ser^2, L-Leu^5-enkephalyl-Thr (DSLET) (Garcel et al. 1980). Highest selectivity for the δ receptor is exhibited by the conformationally restricted, cyclic, disulfide-containing an logs [2-D-penicillamine, 5-L-penicillamine]enkephalin ([D-Pen^2, L-Pen^5]enkephalin), and [2-D-penicillamine, 5-D-penicillamine]enkephalin ([D-Pen , D-Pen^5]enkephalin) (Mosberg et al., 1983). In binding assays, [D-Pen^2, L-Pen^5] and D-Pen^2,D-Pen^5]enkephalin exhibited δ:μ receptor selectivities of 371 and 175, respectively; compared with the other δ analogs, these bis-Pen-containing analogs provide an order of magnitude increase in δ receptor selectivity. Thus, although most μ and δ ligands cross-react with μ and δ receptors, highly selective μ and δ ligands have been designed. Neither μ nor δ opioids exhibit significant affinity for the κ or σ receptors. The prototypic κ ligands ketocyclazocine and ethylketocyclazocine (EKC) do exhibit high affinities for both μ and κ receptors (Eghbali et al., in press; Kosterlitz et al. 1981). More recently, U-50,488 has been

TABLE 1. *Binding parameters for opioid receptor sites in rat striatum*

Receptor Type	K_d1 (nM)	$B_{Max}1$ (fmol/mg)	K_d^2 (nM)	B_{Max}^2 (fmol/mg)
μ Receptors	1.7	108		
δ Receptors	7.1	184		
Total [^{3}H]EKC Sites*	0.7	94	8.7	252
κ Receptors**	8.2	74		

*Total specific [^{3}H]EKC binding was carried out In the absence of μ and δ blockers.

**κ receptor binding was carried out using [^{3}Hl]EKC In the presence of DAGO and DADLE, which block binding of radioligand to μ and δ receptors, respectively.

reported to be a highly selective κ ligand essentially devoid of μ or δ activity (VonVoigtlander et al. 1983). Prototypic μ agonists ((-)SKF-10,047 and (-)cyclazocine) exhibit potent affinities at μ and, in the case of cyclazocine, κ receptors (Goodman and Snyder 1982; Zukin and Zukin 1981). Phencyclidine and (+)SKF-10,047 are specific ligands for the σ receptor (Zukin and Zukin 1981; Zukin and Zukin 1984) and exhibit no detectable affinity for μ, δ, or κ receptors.

A variety of studies indicate that μ and δ receptors have somewhat different distributions throughout the central and peripheral nervous systems. Thus, the thalamus and hypothalamus of rat brain (Chang et al. 1979) and guinea pig ileum (Lord et al. 1977) appear to be highly enriched in μ receptors, whereas the rat frontal cortex, rat corpus striatum (Chang et al. 1979), and mouse vas deferens (Lord et al. 1977) show relatively higher densities of δ receptors. Of particular importance has been the finding of tissues homogeneous in δ receptors, such as the neuroblastoma cell line N4GT1 (Chang et al. 1978). The distributions of μ and δ receptors have been confirmed in the central nervous system (CNS) by light microscopy autoradiography (Goodman et al. 1980) and in the peripheral nervous system (PNS) by the twitch and other bioassays on isolated tissue strips (Chang et al. 1978; Robson and Kosterlitz 1979). These studies, however, did not use specific radioligands to label μ or δ receptors. Goodman and Snyder (1982) used [^{3}H]EKC and [^{3}H]bremazocine (another κ drug) in the presence of selective μ and δ blockers in an autoradiography study to elucidate a unique κ receptor distribution in guinea pig brain. The highest selective localization of putative κ receptors was found to occur in layers V and VI of the cerebral cortex. These researchers did not, however, study the κ receptor distribution in rat brain.

The present chapter reviews <u>in vitro</u> receptor binding studies and quantitative receptor autoradiography studies using highly specific radioligands to label μ, δ, and κ receptors. The relative densities and affinities of the three receptor classes in rat brain, as well as their neuroanatomical distribution patterns, are discussed. These studies reveal strikingly different patterns for the three opioid receptors.

EQUILIBRIUM BINDING STUDIES OF μ, d, AND k RECEPTORS IN RAT BRAIN MEMBRANES

Binding parameters for μ, δ, and κ opioid receptors can be determined by incubating brain membranes with radioligands highly specific for the various receptor classes.Striatal tissue in our studies was incubated with [3H]D-Ala^2, N-Me-Phe^4, Gly-ol^5-enkephalin (DAGO) (for μ receptor assays), [^{3}H][D-Pen2, D-Pen^2]enkephalin (for δ receptor assays), or [^{3}H]ethylketocyclazicine (EKC) (for κ receptor assays) in 50 mM Tris-HCl buffer, pH 7.4, at 4 °C for 60 minutes in the absence or presence of the same nonradioactive ligand (10 μM). Radioligand concentration was varied over a concentration range of 0.1 to 25 nM. In the case of κ receptor assays,

binding was carried out in the presence of DAGO (100 nM) and DADLE (100 nM) in order to block the binding of [^{3}H]EKC to μ and δ receptors and ensure its Specific binding to κ receptors (Eghbali et al., in press). This concentration was determined to be more than 100 times greater than the IC_{50} for either ligand in displacement of [^{3}H]EKC, in confirmation of Kosterlitz et al. (1981).

Figure 1 shows aturation and Scatchard analyses of the equilibrium binding of [^{3}H]DAGO (μ-specific opioid, 0.1 to 25 nM) to rat striatal membranes. Scatchard analysis revealed a linear plot (figure 1). Computer-assisted linear regression analysis (Munson and Rodbard 1980) afforded a best fit for a single binding component; the calculated parameters were K_d=1.72 nM, B_{Max}=I08 fmol/mg protein. Figure 2 depicts representative saturation and Scatchard isotherms for [^{3}H][D-Pen^2, D-Pen^5]enkephalin (δ-specific ligand, 0.1 to 25 nM) binding to striatal membranes. Scatchard analysis of δ receptor binding again yielded a straight line, the calculated parameters Of which were K_d=10.5 nM and B_{Max}=142 fmol/mg protein (figure 2).

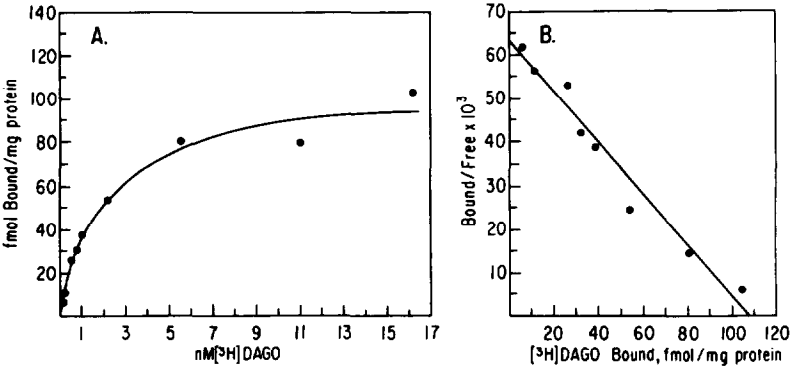

FIGURE 1. *Saturation (A) and Scatchard (B) analyses of specific [^{3}H]DAGO binding to rat striatal tissie*

NOTE: Aliquots of homogenate (250 μl, 0.9 to 1 mg/ml protein) In 50 ml Tris-HCl buffer. pH 7.4, were Incubated In triplicate at 4 °C for 60 minutes with [^{3}H]DAGO (0.1 to 20 nM). In the absence or presence of 10 μM nonradiolabelled DAGO. Free [^{3}H]DAGO was separated from bound ligand by the rapid filtration method. Specific binding, defined as total binding minus binding in the presence of nonradioactive DAGO, is reported. Data as shown were fit by straight lines using computer-assisted linear regression analysts. This experiment was replicated two times.

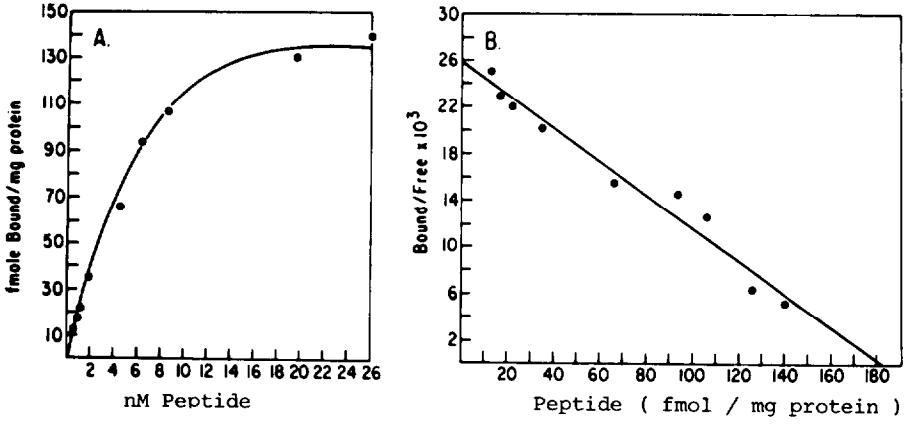

FIGURE 2. *Saturation (A) and Scatchard (B) analyses of specific [^{3}H][D-Pen^2, D-Pen^5]enkephalin binding to rat striatal tissue*

NOTE: Aliquots of homogenate (250 µl. 0.9 to 1 mg/ml protein) in 50 mM Tris-HCl buffer, pH_2 7.4, were incubated In triplicate at 4 °C for 3 hours with [^{3}H], [D-Pen^2 , D-Pen^5]enkephalin (0.1 to 20 nM) in the absence or presence of 10 µM nonradiolabel led ligand. Separation of free ligand from bound ligand and data analysis were carried out as described In the legend to figure 1. Th Is experiment was replicated two times.

κ receptors can be labelled specifically in preparations of rat brain membranes using the κ opioid [^{3}H]EKC in the presence of non-labelled DAGO and DADLE. These peptides serve to block binding of the radioligand to µ and δ receptors, respectively, and direct its binding to κ receptors (Kosterlitz et al. 1981; Goodman and Snyder 1982; Eghbali et al., in press). Scatchard analysis of specific [^{3}H]EKC binding (unblocked) to rat striatal membranes revealed a biphasic curve suggesting binding to at least two classes of binding sites (figure 3). Computer-assisted nonlinear regression analysis (Munson and Rodbard 1980) afforded a best fit for a curve calculated for two binding components. The first was characterized by κ=0.94±0.14 nM and B_{Max} =94 fmol/mg protein; the second, by K_d=8.6±2.4 nM and B_{Max}=±1 fmol/mg protein.

Experiments in which [^{3}H]EKC binding was determined in the presence of DAGO (100 nM) and DADLE (100 nM) yielded a linear Scatchard plot for rat brain membranes (K_d=11.4±1.9 nM and B_{Max}=119±19 fmol/mg protein) (figure 4). That these sites are likely to be pharmacologically relevant κ receptors was established in guinea pig brain tissue by Kosterlitz and coworkers (Kosterlitz et al. 1981). They demonstrated that the rank order of potencies of a large series of opioids in inhibiting binding of

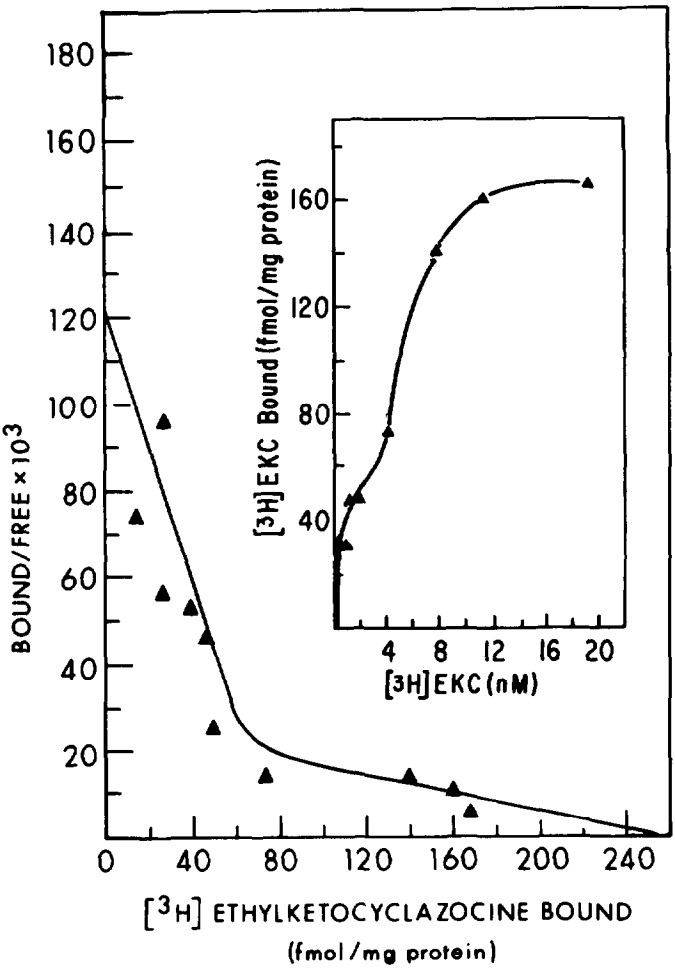

FIGURE 3. *Scatchard analysis of specific [^{3}H]ethylketocyclazocine (EKC) biding to rat brain membranes*

NOTE: Aliquots of homogenate (1 ml, 0.9 mg protein) in 50 nM Tris-HCl buffer, pH 7.4, were incubated in triplicate at 4 °C for 60 minutes with various concentrations of [^{3}H]EKC in the absence or presence of 10 µM nonradioactive EKC. Free [^{3}H]EKC was separated from bound ligand by the rapid filtration method as described In the legend to figure 1. Specific binding, defined as total binding minus binding In the presence of nonradioactive EKC, is reported. Data as shown were fit by straight lines using computer-assisted linear regression analysis. The biphasic plot was analyzed using computer-assisted nonlinear least squares regression analysis (Munson and Rodbard 1980). This experiment was replicated two times.

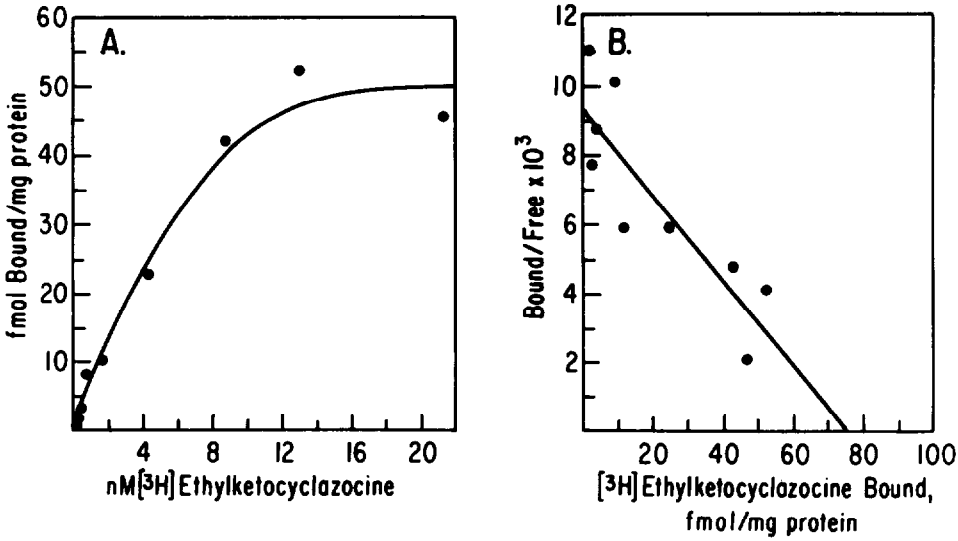

FIGURE 4. *Saturation (A) and Scatchard (B) analyses of specific [^{3}H]EKC binding to rat brain homogenate in the presence of DAGO (μ-selective opioid) and DADLE (δ-selective opioid)*

NOTE: Rat brain homogenates were Incubated in triplicate for 60 minutes under the same experimental conditions as described In the legend to figure 3, except that μ and δ receptor binding was suppressed by the Inclusion of DAGO (100 nM) and DADLE (100 nM), respectively. Data as shown were fit by straight lines using computer-assisted linear regression analysis. This experiment was replicated two times.

[^{3}H]EKC to these sites paralleled that found for behavioral and pharmacological assays of κ activity. Inclusion of the cerebellum in the brain homogenate did not result in any significant change in either K_d or B_{Max} values. [^{3}H]EKC binding to rat brain in the presence of U-50,488H, a highly specific κ ligand, revealed a monophasic Scatchard plot with a K_d similar to the high-affinity component. Table 1 summarizes μ, δ and κ receptor affinities and receptor densities obtained in rat brain tissue.

AUTORADIODRAPHIC STUDIES OF μ, δ, AND κ DPIOID RECEPTORS

In order to visualize the neuroanatomical distribution patterns of the μ, δ, and κ opioid receptors, autoradiography at the level of the light microscope was carried out on thaw-mounted sections of frozen rat brain. Autoradiograms of selected coronal sections are shown in figures 5 through 7. μ receptor labelling at the level of the anterior commissure revealed strikingly dense patches of

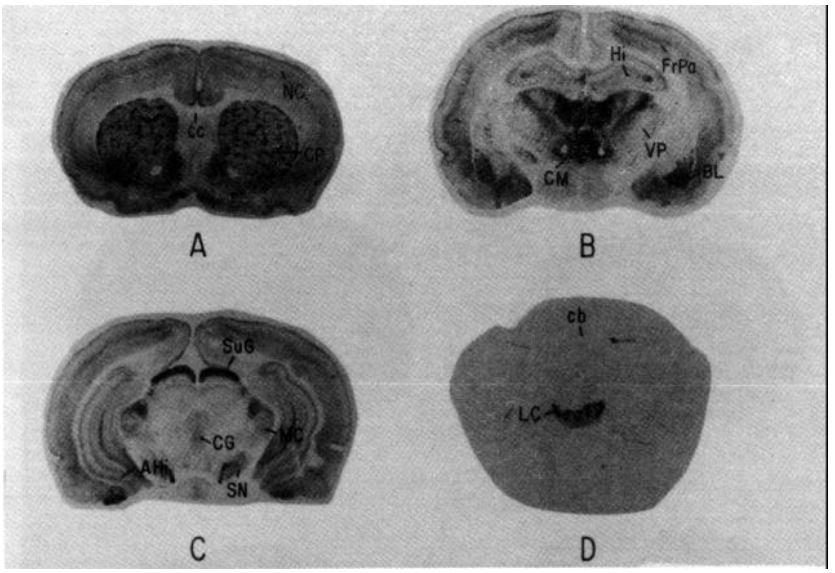

FIGURE 5. *Photomicrographs of [^{3}H]D-Ala^2, N-Me-Phe^4, Gly-ol^5-enkephalin (DAGO) autoradiograms of selected coronal sections of rat brain*

KEY: CP=caudate; NC=neocortex; CC=corpus callosum; HI-hippocampus; FrPa=frontal parietal cortex; BL=basolateral amygdaloid nucleus; VP=ventroposterior thalamic nucleus; CM=central medial thalamic nucleus; SuG=gray layer of superior colliculus; MC=medial geniculate nucleus; CG=central gray; SN=substantia nigra; AHI=amygdalohippocampal area; cb-cerebellum; LC=locus coeruleus.

NOTE: Brain sections were labelled in vitro In a solution of 4 nM [^{3}H]DAGO (60 Cl/mmol). To assess nonspecific binding, adjacent sections were Incubated under the same conditions in a solution containing radiolabelled ligand in the presence of a 1,000-fold excess of levorphanol. Sections corresponding to both "total binding" and "nonspecific binding" from each brain region were exposed to the same sheet of LKB film together with tritium standards (Amersham). After appropriate exposure time (8 to 12 weeks). the film was developed using standard Kodak developer (D-19) and fixative (5 minutes D-19, 20 seconds H_2O rinse, 10 minutes fixative). The optical density of each structure was determined by a Photovolt densitometer with an aperture diameter of 0.1 mm (Photovolt Corp., New York, NY). For each brain structure, densitometric readings were taken from a minimum of three consecutive sections per rat after correction for the contributions due to nonspecific binding and background film density. Corrected densitometric readings (In optical density units) were then averaged and converted to receptor density values (fmol/mg) by reference to a standard curve for brain tissue computed using tritium standards (Amersham). Receptor density values reported are means ± S.D. of femtomoles of radlolabelled ligands, specifically bound per milligram of tissue, of averaged values from the corresponding frozen sections of a minimum of these rats. Figure 5A depicts μ receptor labelling at the level of the anterior commissure. Dense patches overlying the striosomes of the caudate putamen can be seen surrounded by diffusely organized receptors. Figure 58 depicts μ receptor binding at the level of the diencephalon. In the thalamic nuclei, μ receptor labelling can be seen specifically in the medial and midline nuclear groups. In the midbrain area of rat brain (figure 5C), a hlgh density of μ receptors was observed In the substantia nigra reticulata, the interpeduncular nucleus, and the dorsal lateral aspects of the central gray. [^{3}H]DAGO blndlng to the rat brainstem (figure 5D) revealed dense labelling In the locus coeruleus of the pons.

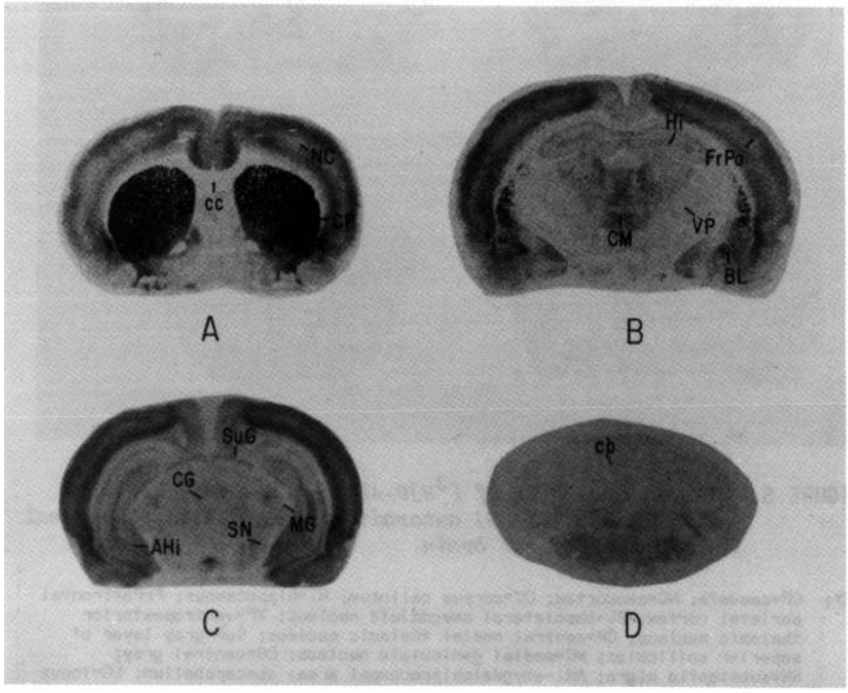

FIGURE 6. *Photomicrographs of selected coronal sections demonstrating δ receptor labelling in rat brain*

NOTE: δ receptors were labelled using 6 nM [^{3}H]D-Pen^2, D-Pen^5]enkephalin (41.5 Cl/mmol). Procedures for receptor labelling and for autoradiography were as described in the legend to figure 5. Figure 5A depicts δ receptor labelling et the level of the telencephalon. δ receptors exhibit few patches In the caudate putamen and a dense, diffuse distribution throughout the striatum. [^{3}H]DPDPE labelling at the level of the neocortex (figure 58) revealed heaviest labelling In layers I, II, V, and VI. Note the lack of δ receptors in nuclei of the thalamus and hypothalamus (B), midbrain (C), and hindbrain (D). For the key to the abbreviations, see the legend to figure 5.

receptors overlying the striosomes (figure 5A). These patches were surrounded by areas of diffusely organized receptors. This topographical organization is consonant with the previously reported pattern of μ receptor labelling in the striatum (Tempel et al. 1984; Herkenham and Pert 1982; Pert et al. 1976; Atweh and Kuhar 1977). μ receptor labelling in the neocortex of rat brain (figure 5A) revealed a highly specific pattern corresponding to known cytoarchitectural boundaries. Labelling was of highest

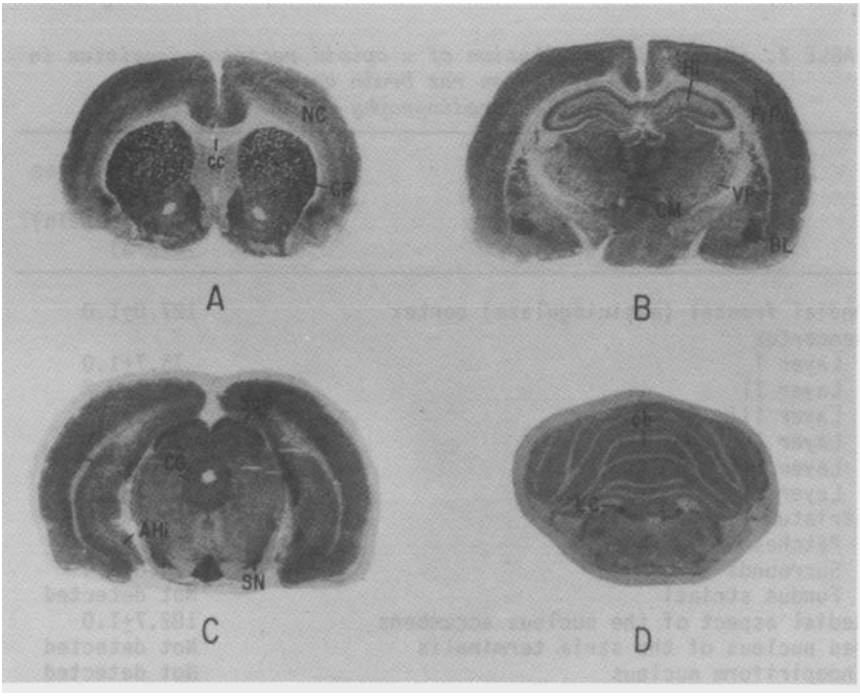

FIGURE 7. *Photomicrographs of [^{3}H]EKC autoradiograms of selected coronal sections of rat brain*

NOTE: Thaw-mounted sections of frozen rat brain were prepared as described In the legend to figure 5. Sections were Incubated in 50 nM Tris-HCI (pH 7.4, 4 °C) containing 10 nM [^{3}H]EKC In the presence of 100 nM DAGO and 100 nM DADLE to block binding of the radloligand to μ and δ receptors and direct its binding to κ receptors. To assess nonspecific binding, adjacent sections were Incubated under the same conditions in a solution containing radlolabelled ligand in the presence of a 1,OOO-fold excess of EKC (10 μM). κ receptor labelling can be seen In (A) the striatal area and the bed nucleus of the stria terminalis and (B) the hippocampal formatIon, especially overlylng the pyramidal cell field and the granular cell layer. Note the dense labelling in several of the thalamic nuclei, including the medial and midline nuclear groups. Also seen In thls autoradiogram is the neocortex, which is uniformly labelled by [^{3}H]EKC. κ receptor labelling can be seen in (C) the midbrain area, particularly In the substantia nigra reticulata. the interpeduncular nucleus, and the central gray. Dense κ receptor labelling can also be seen in (D) the locus coeruleus, the cerebellum, and the spinal tract of the trigeminal nerve of the brainstem. For abbreviations, see the legend to figure 5.

density in layers I and III of the cortex. Dense μ receptor labelling was also notable in the cingulate cortex and adjacent medial frontal cortex. A particularly high density of receptors was observed in the ventral striatal and pallidal areas, endopiriform nucleus, and diagonal band of Broca. By contrast, μ receptor labelling was noticeably lacking in the anterior commissure and corpus callosum. Table 2 summarizes μ receptor densities in rat brain structures, as determined by quantitative, in vitro autoradiography.

TABLE 2. *Regional distribution of μ opioid receptor densities in sections of frozen rat brain as determined by quantitative autoradiography*

Region	μ Receptor Densities [^{3}H]DAGO (fmol/mg protein) (n=3)
Medial frontal (anticingulate) cortex	127.0±1.0
Neocortex	
Layer I	75.7±1.0
Layer II	28.7±0.2
Layer III	100.9±3.2
Layer IV	28.7±0.2
Layer V	28.7±0.2
Layer VI	28.7±0.2
Striatum	
Patches	226.2±7.1
Surrounds	114.8±3.4
Fundus striati	Not detected
Medial aspect of the nucleus accumbens	182.7±1.0
Bed nucleus of the stria terminalis	Not detected
Endopiriform nucleus	Not detected
Amygdala	Not detected
Posteromedial cortical	67.9±0.3
Basolateral nucleus	100.9±1.1
Lateral septum	127.0±2.2
Hippocampus	
Molecular layer	52.2±2.1
Pyramidal cell layer	22.6±1.9
CA_2	55.7±1.1
CA_3	28.7±0.1
Thalamic nuclei (in total)	
Centromedial	95.7±2.2
Reunions	127.0±1.3
Parvocellular area of ventroposterior nucleus	Not detected
Lateral mediodorsal	Not detected
Lateral posterior	75.7±2.1
Anterior laterodorsal	52.2±3.1
Anterior ventral	34.8±2.8

TABLE 2 (Continued)

Region	μ Receptor Densities [^{3}H]DAGO (fmol/mg protein) (n=3)
Posteromedial cortical	Not detected
Posterior nucleus	74.0±0.1
Centrolateral	132.2±11.2
Intermediodorsal	Not detected
Rhomboid	132.2±7.7
Medial geniculate	Not detected
Dorsal lateral geniculate	63.3±0.1
Hypothalamic nuclei	Not detected
Dorsal	Not detected
Ventromedial	Not detected
Medial habenulae	121.8±1.3
Substantia nigra reticulata	63.5±0.2
Interpeduncular nucleus	55.7±0.1
Superficial gray layer of the superior colliculus	83.5±0.2
Inferior colliculus, medial portion	104.4±0.2
Central gray	38.3±0.1
Locus coeruleus	92.2±7.0
Cerebellum	Not detected
Spinal tract of the trigeminal nerve	Not detected
Corpus callosum	9.1±0.1

NOTE: μ opioid receptors were labelled as described in the legend to figure 5. For each brain structure, densitometric readings were taken from a minimum of three consecutive sections per rat after correction for contributions due to nonspecific binding and background film density. Corrected densitometric readings (in optical density units) were then averaged and converted to receptor density values (fmol/mg) by reference to a standard curve for brain tissue, computed using tritium standards (Amersham). Receptor density values reported are means ± S.E.M. of femtomoles of [^{3}H]DAGO specifically bound per milligram of tissue, of averaged values from the corresponding frozen sections of a minimum of three rats.

Figure 5B depicts μ receptor binding to a section of frozen rat brain at the level of the diencephalon. μ receptors were highly concentrated in the pyramidal cell field, especially in the CA_2 region of the hippocampal formation.

In the thalamic area, dense μ receptor labelling was localized to specific nuclei (figure 5C) as follows: the lateral mediodorsal, lateral posterior, anterior laterodorsal, and posterior nuclei, and the medial and midline nuclear groups, including the centromedial nucleus, centrolateral nucleus, reunions nucleus, and rhomboid nucleus.

At the level of the midbrain, regions exhibiting a high density of μ receptors were the medial aspect of the inferior colliculus, the superficial gray layer of the superior colliculus, and the substantia nigra reticulata. The interpeduncular nucleus and the dorsal lateral aspects of the central gray exhibited a somewhat lower level of μ receptor labelling. In the brainstem region (figure 50) the locus coeruleus of the pons revealed a particularly dense clustering of this receptor type. All other areas were devoid of μ receptors.

Figure 6A depicts the pattern of δ receptors labelled by [^{3}H][2-D-Pen, 5-D-Pen]enkephalin in the telencephalon at the level of the striatum (caudate putamen). Characteristically, δ receptors exhibited few patches and a diffuse distribution throughout the striatum with more dense labelling in the ventrolateral regions and fundus striati. δ receptor labelling was absent from the anterior commissure and corpus callosum. [^{3}H]DPDPE labelling at the level of the neocortex revealed a pattern distinctly different from that of μ receptors. Highest labelling was observed in layers I, II, V, and VI. No δ receptors were detected in layers III or IV of the cortex. Nuclei of the thalamus, hy thalamus, midbrain, and brainstem were noticeably lacking in [^{3}H]DPDPE-labelled receptors. Table 3 summarizes δ receptor densities in these rat brain structures as determined by quantitative, in vitro autoradiography.

TABLE 3. *Regional distribution of δ opioid receptor densities in sections of frozen rat brain, as determined by quantitative autoradiography*

Region	δ Receptor Densities [^{3}H]DPDPE (fmol/mg protein) n = 3
Medial frontal (anticingulate) cortex	
Neocortex	
Layer I	79.5±5.0
Layer II	79.5±5.0
Layer III	Not detected
Layer IV	Not detected
Layer V	132.6±9.1
Layer VI	136.6±9.1
Striatum	
Patches	Not detected
Surrounds	169.9±2.1
Fundus striati	192.8±11.2
Medial aspect of the nucleus accumbens	125.3±1.3
Bed nucleus of the stria terminalis	Not detected
Endopiriform nucleus	Not detected
Amygdala	Not detected

TABLE 3 (Continued)

Region	δ Receptor Densities [^{3}H]DPDPE (fmol/mg protein) (n=3)
Posteromedial cortical	Not detected
Basolateral nucleus	Not detected
Lateral septum	Not detected
Hippocampus	
Molecular layer	Not detected
Pyramidal cell layer	Not detected
CA_2	Not detected
CA_3	Not detected
Thalamic nuclei (in total)	
Centromedial	Not detected
Reunions	Not detected
Parvocellular area of the ventroposterior nucleus	Not detected
Lateral mediodorsal	Not detected
Lateral posterior	Not detected
Anterior laterodorsal	Not detected
Anterior ventral	Not detected
Posteromedial cortical	Not detected
Posterior nucleus	Not detected
Centrolateral	Not detected
Intermediodorsal	Not detected
Rhomboid	Not detected
Medial geniculate	Not detected
Dorsal lateral geniculate	Not detected
Hypothalamic nuclei	Not detected
Dorsal	Not detected
Ventromedial	Not detected
Medial habenulae	Not detected
Substantia nigra reticulata	Not detected
Interpeduncular nucleus	Not detected
Superficial gray layer of the superior colliculus	Not detected
Inferior colliculus, medial portion	Not detected
Central gray	Not detected
Locus coeruleus	Not detected
Cerebellum	Not detected
Spinal tract of the trigeminal nerve	Not detected
Corpus callosum	3.3±0.4

NOTE: δ opioid receptors were labelled as described In the legend to figure 6. Densitometric readings and quantification of autoradiograms were performed as described In the legend to table 2.

In order to visualize the neuroanatomical pattern of κ opioid receptors, light microscopy autoradiography was carried out on thaw-mounted sections of frozen rat brain. Figure 7 shows LKB film autoradiograms of [^{3}H]EKC binding after suppression of μ and δ binding in selected coronal sections. Figure 7A shows an autoradiogram at the level of the telencephalon; a very low density of κ sites was found to overlie the striosomes or patches of the striatum. By contrast, dense κ receptor labelling was identified with the bed nucleus of the stria terminalis. In the neocortex (figures 7A and 7B), κ receptor labelling was nearly uniform throughout the molecular layers; the pattern of receptor labelling failed to correspond to known cytoarchitectural boundaries. By contrast, κ receptor labelling in guinea pig neocortex (Goodman and Snyder 1982; Eghbali et al., in press) exhibits a highly specific pattern of intense labelling in layers V and VI of the neocortex.

Figure 7B depicts κ binding to a section of frozen rat brain at the level of the diencephalon. A high density of κ receptors was found to overlie the pyramidal cell layers adjacent to the molecular layers of the hippocampal formation, CA_1, CA_2, and CA_3 areas, and the granular layer of the dentate gyrus. By contrast, κ receptors in guinea pig brain sections are of high density only in the molecular layer of the dentate gyrus and the pyramidal cell layer of the hippocampal formation (Eghbali et al., in press).

In the thalamic and hypothalamic nuclei, striking differences were seen between the rat and the guinea pig. In the thalamus of the rat brain, only selected nuclei exhibited a high density of κ receptors (figure 7B); these were the laterodorsal nucleus, the posterior nucleus, and the medial and midline nuclear groups (for example, the centrolateral nucleus, intermediodorsal nucleus, rhomboid nucleus, and the gelatinosus nucleus of the thalamus). Moderate levels of κ receptor labelling were seen in the hypothalamic area, for example, the dorsal hypothalamic area and ventromedial nucleus of the hypothalamus. By contrast, the guinea pig brain displays only a low level of κ receptor labelling uniformly distributed throughout the thalamic and hypothalamic nuclei. A notable exception is the medial geniculate nucleus of the thalamus, which exhibits a dense concentration of κ receptors (Eghbali et al., in press).

In the midbrain area of the rat brain (figure 7C), examples of regions exhibiting high densities of κ receptors were the periaqueductal gray (primarily the dorsal area), the substantia nigra reticulata, and the interpeduncular nucleus. Moreover, the superior colliculus exhibi ed dense κ receptor labelling in the superficial gray layer. [^{3}H]EKC binding to the rat brainstem at the level of the locus coeruleus (figure 7D) revealed dense labelling in the locus coeruleus, the central gray area of the pons, and the cerebellum. Moderate levels of κ receptors were also seen in the spinal tract of the trigeminal nerve (fifth nerve). Table 4 summarizes κ receptor densities in rat brain structures, as determined by quantitative, in vitro autoradiography.

TABLE 4. *Regional distribution of κ opioid receptor densities in sections of frozen rat brain, as determined by quantitative autoradiography*

Region	κ Receptor Densities in Rat Brain (fmol/mg) (n=3)
Neocortex	
Layers I-IV	41.9±2.1
Layers V-VI	41.9±2.1
Striatum	
Patches	71.0±5.0
Surrounds	45.5±2.3
Nucleus accumbens	58.2±3.5
Bed nucleus of the stria terminalis	81.9±7.4
Hippocampus	
Molecular layer	Not detected
Pyramidal cell layer	41.9±1.7
Granular cell layer	51.0±2.6
Thalamic nuclei (in total)	
Laterodorsal nucleus	56.4±0.2
Posterior nucleus	72.8±2.9
Centrolateral nucleus	83.7±3.4
I ntermediodorsal nucleus	98.3±4.9
Rhomboid nucleus	92.8±9.3
Medial geniculate nucleus	Not detected
Hypothalamic nuclei	
Dorsal hypothalamic area	51.0±1.0
Ventromedial nucleus	56.4±3.4
Substantia nigra reticulata	51.0±1.3
Interpeduncular nucleus	72.8±2.2
Superficial gray layer of the superior colliculus	78.3±3.1
Central gray	56.4±1.1
Locus coeruleus	78.3±8.6
Cerebellum	32.8±1.0
Spinal tract of the trigeminal nerve	43.7±1.8
Corpus callosum	7.3±1.0

NOTE: κ opioid receptors were labelled as described In the legend to figure 7. Densitometric readings and quantification of autoradiograms were performed as described in the legend to table 3.

Autoradiographic studies using highly specific radiolabelled opioids clearly demonstrate strikingly different neuroanatomical distribution patterns for the μ and δ receptors in rat brain (Pert et al. 1976). μ receptors appear to be most densely localized in the patches of the striatum, layers I and III of the cortex, the pyramidal cell layer of the hippocampal formation, specific nuclei

of the thalamus, the substantia nigra, the interpeduncular nucleus, and the locus coeruleus of the pons. δ receptors labelled by the specific δ ligand [^{3}H]DPDPE exhibit selective localization in layers I, II, V, and VI of the cortex and a dense, diffuse pattern in the striatum with no visible patches. δ receptors are notably absent in most other brain structures.

The present study further demonstrates, on the basis of both in vitro binding assays and quantitative receptor autoradiography at the level of the light microscope, the presence of κ binding sites in rat brain. Although κ receptors in rat and guinea pig brain exhibit similar distributions in the midbrain and hindbrain, they exhibit strikingly different patterns in several forebrain structures. First, in sections of rat brain, κ receptor labelling exhibits dense patches in the corpus striatum and a dense concentration in the nucleus accumbens, whereas in guinea pig brain it exhibits a low, uniform labelling in the striatum and a low level of density in the nucleus accumbens. Secondly, κ receptors exhibit selective localization in the deep layers (V and VI) of the guinea pig neocortex, whereas in the rat neocortex, κ receptors exhibit no visible boundaries corresponding to cytoarchitectural boundaries. Thirdly, in the rat hippocampal formation, κ receptors show highest selective localization in the pyramidal and granular cell layers, whereas in the guinea pig (Eghbali et al., in press), κ receptor labelling is most dense in the pyramidal cell layer and the molecular layer of the dentate gyrus. Finally, the most striking differences between the species are seen in the thalamic and hypothalamic areas. In rat brain, dense κ receptor labelling is seen in the central and midline nuclei of the rat thalamus, and moderate labelling, in certain hypothalamic nuclei. By contrast, in guinea pig brain, a significant density of κ receptors is observed only in the medial geniculate nuclei of the thalamus (Eghbali et al., in press). It is interesting to note that the oldest parts of the brain (the hindbrain and midbrain areas) share commonalities of κ receptor distribution in the two species. The more recently developed areas of the forebrain show species-specific distribution patterns of κ receptors, which may correlate with differences in the ontogeny of the various opioid receptor types.

κ receptor labelling was strikingly similar to μ receptor labelling. Dense labelling was seen in the patches of the striatum, the nucleus accumbens, the pyramidal cell layer of the hippocampal formation, the midline nuclei of the thalamus, and the locus coeruleus and cerebellum of the brainstem. It is interesting that the distribution patterns of the μ and κ receptors share many commonalities in the rat brain. The time course of their appearance in the developing brain could account for many of these similarities. The δ receptors appear to be localized to the more recently developed areas of the forebrain, such as the neocortex and striatum, which may indicate a more advanced need for specialized receptors either as modulators or for specialized functions.

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Anatomical and Biochemical Perspectives on Opioid Peptides

Robert P. Elde

INTRODUCTION

A decade has now passed since the initial report describing the isolation, structural characterization, and synthesis of the opioid peptides leucine and methionine enkephalin (Hughes et al. 1975). The subsequent discovery of other opioid peptides (i.e., ß-endorphin (Li and Chung 1976; Ling et al. 1976) and the dynorphins (Goldstein et al. 1979; Goldstein et al. 1981)) led to numerous imnunocytochemical studies describing the distribution of these peptides. This information has established the notion that the physiologically relevant occupants of opioid receptors are produced by discrete neuronal circuits that use opioid peptides as intercellular messengers. The purpose of this chapter is to review some of the features of the chemical neuroanatomy of opioidergic neurons. In addition, an attempt will be made to identify some of the fundamental problems that remain concerning the neurobiology of opioid neurons.

STRUCTURAL FEATURES OF OPIOID PEPTIDES

The discoveries of extended forms of met-enkephalin (i.e., a-endorphin and ß-endorphin (Guillemin et al. 1976; Li and Chung 1976; Ling et al. 1976) and BAM-22P (Mizuno et al. 1980)) as well as extensions of leu-enkephalin (i.e., dynorphin, α-neo-endorphin, and rimorphin (Goldstein et al. 1979; Goldstein et al. 1981; Kilpatrick et al. 1982)) initially led to confusion among investigators, because it was not clear which might be the most important opioid peptide. This confusion did not last long, however, because immunohistochemical and radioimmunoassay studies quickly suggested a differential localization of many of these peptides, implying an independent existence of the enkephalins from ß-endorphin (Watson et al. 1978) and from the several forms of dynorphin (Watson et al. 1982; Watson et al. 1983; Weber et al. 1982a; Weber et al. 1982b; Weber et al. 1982c). Proof of the existence of discrete families of opioid peptides came from the cloning and sequencing of DNA complementary to messenger RNA for preproenkephalin (figure 1) (Gubler et al. 1981; Noda et al. 1982a; Noda et

al. 1982b); preproopiomelanocortin (figure 2) (Nakanishi et al. 1979; Nakanishi et al. 1981; Roberts et al. 1979); and prepro-dynorphin (figure 3) (Kakidani et al. 1982).

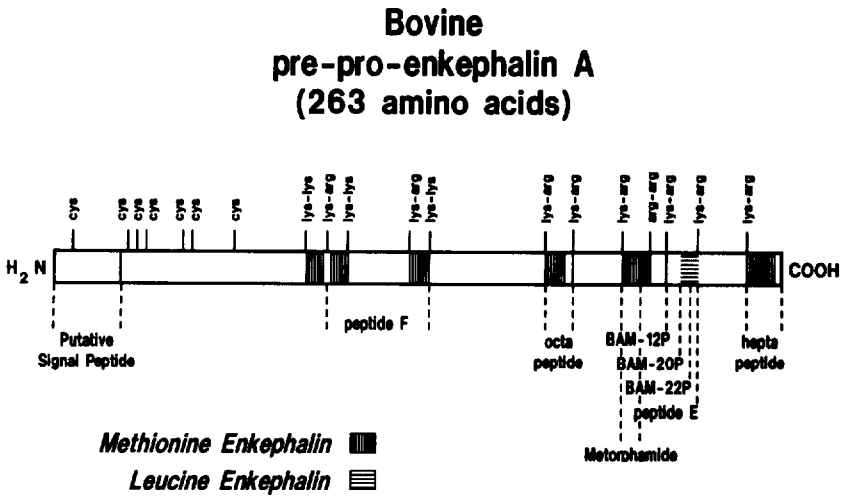

FIGURE 1. *Schematic of the proposed structure of bovine preproenkephalin deduced from complementary DNA sequencing*

SOURCE: Gubler et al. (1981); Noda et al. (1982a); Noda et al. (1982b).

The principles of posttranslational cleavage of precursor proteins (Loh and Gainer 1983) (see below) suggest that the opioid peptides isolated and sequenced from various tissues arise from one or more of these precursors. Nevertheless, it remains possible that other independent opioid peptides, as well as precursor proteins, will be identified in future studies. In addition, the possible existence and synthesis of endogenous alkaloid opioids in brain has recently received experimental support (Goldstein et al. 1985).

The intraneuronal mechanisms for the synthesis, packaging, processing, and transport of neuropeptides (including the opioids) are distinct from those involved in the production of "classical" neurotransmitters (Hokfelt et al. 1980; Loh and Gainer 1983). In particular, the synthesis of neuropeptides is accanplished by translation of messenger RNA by ribosomally directed precursor protein synthesis. The nascent protein chain is inserted via the "signal" peptide sequence into the cisternae of the rough endoplasmic reticulum with which the ribosomes are associated. Since rough endoplasmic reticulum is restricted to the neuronal cell body and its proximal dendrites (Peters et al. 1976), structures such as axons and their terminals are incapable of supporting the

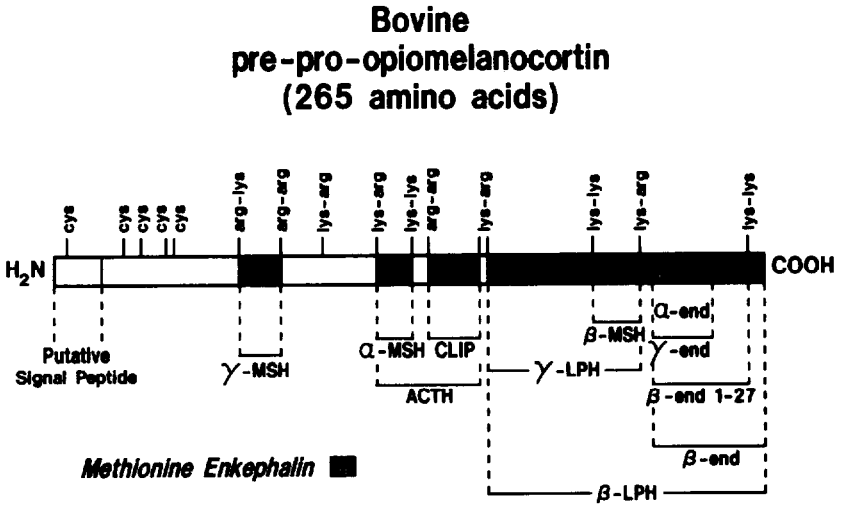

FIGURE 2. *Schematic of the proposed structure of bovine prepro-opiomelanocortin deduced from complimentary DNA sequencing*

SOURCE: Nakanishi et al. (1979); Nakanishi et al. (1981); Roberts et al. (1979).

synthesis of neuropeptides. Thus, the presumed major site of release of neuropeptides--the axon terminal--obtains its stores of peptides only through mechanisms of axonal transport. These processes contrast sharply with the means by which neurons synthesize and store the low molecular weight transmitters such as the monoamines (Cooper et al. 1978). In general, low molecular weight transmitters are synthesized by enzymatic conversion of a precursor molecule into the transmitter. This process may occur anywhere within the neuron in which the enzyme, substrate, and cofactors are present in adequate concentrations and, in contrast to the synthesis of neuropeptides, is thought to occur predominantly in axons and their terminals.

POSTTRANSLATIONAL PROCESSING OF OPIOIDS

The biological activity of opioid peptides is manifest only after cleavage of the appropriate peptide bonds within the precursor molecule liberates the active opioid peptide sequence from neighboring, cryptic peptide sequences. Characterization of the enzymes responsible for these cleavages is currently under intensive investigation (Fricker and Snyder 1983; Goodman et al. 1983). It has been suggested that all the proteolytic enzymes responsible for particular types of cleavages are not uniformly present in cells that produce a given opioid precursor (Weber et al. 1982a; Zakarian and Smyth 1982; Akil et al. 1984; Zamir et al. 1984a; Zamir et al. 1984b; Liebisch et al. 1986). Thus, it is possible that a given precursor can give rise to different opioid products,

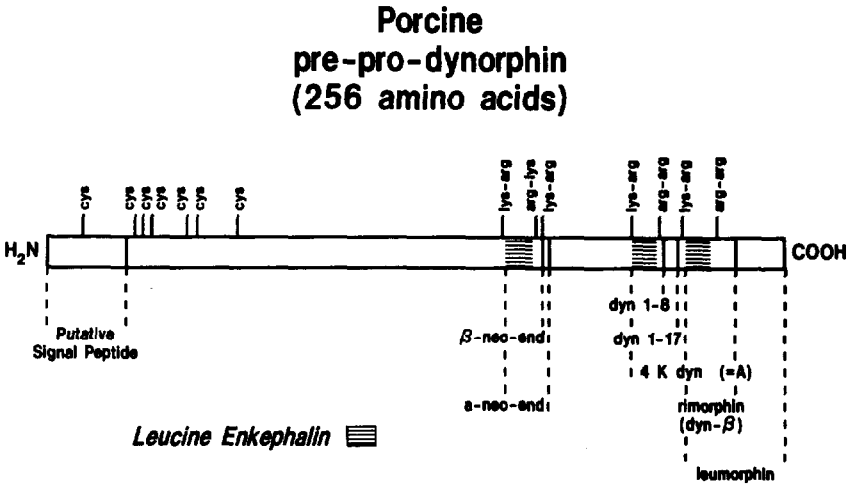

FIGURE 3. *Schematic of the proposed structure of porcine prepmdynorphin deduced from complimentary DNA sequencing*

SOURCE: Kakidani et al. (1983).

if cells di'fferentially produce enzymes responsible for posttranslational processing. In fact, data suggest differences in posttranslational processing of preproenkephalin between central neurons and adrenal chromaffin cells (Mizuno et al. 1980; Liebisch et al. 1986); of preproopiomelanocortin between anterior and intermediate lobe cells in the pituitary (Akil et al. 1984); and of preprodynorphin between certain brain regions, leading to a relative abundance of dynorphin A 1-8 in some areas and to longer forms of dynorphin in others (Weber et al. 1982a; Zamir et al. 1984b).

Posttranslational modification of opioids is not limited to cleavage of the precursor proteins but also includes several types of covalent modification of the cleaved peptides. These covalent modifications of opioids include the a-acetylation of the amino terminal tyrosine of ß-endorphin (Akil et al. 1981; Akil et al. 1984) and the amidation of the carboxy terminus of metorphamide (Weber et al. 1983). It is important to note that posttranslational modifications of opioids may serve to enhance the biological activity of the molecule (as in cleavage of the biologically active opioids from precursor molecules), alter its receptor selectivity, or inactivate it (Akil et al. 1981; Akil et al. 1984).

LOCALIZATION STUDIES OF OPIOID PEPTIDES IN NEURONS

The ability to produce antisera selective for the various opioid peptides has permitted both quantitative radioimmunoassay surveys of extracts of macroscopic regions of the nervous system (Weber et al. 1982a) and cellular identification of opioid neurons (Elde et al. 1976; Hokfelt et al. 1977; Sar et al. 1978; Watson et al. 1978; Watson et al. 1981; Watson et al. 1982; Uhl et al. 1979; Khachaturian et al. 1982; Khachaturian et al. 1983a; Khachaturian et al. 1983b; Vincent et al. 1982; Weber et al. 1982b; Furness et al. 1983; recently reviewed by Khachaturian et al. 1985). The opioid peptides derived from preproenkephalin and preprodynorphin are widely distributed among neurons of both the peripheral (sympathetic, parasympathetic, and enteric nervous systems) and central nervous systems. In contrast, neurons producing opioids derived from preproopiomelanocortin are not found in the peripheral nervous system at all and are restricted to only two areas of the mammalian central nervous system--the arcuate nucleus of the hypothalamus and the nucleus of the solitary tract within the medulla oblongata. For each family of opioid peptides, the areas containing nerve fibers and terminals that release opioid peptides outnumber the areas containing neuronal cell bodies, thus suggesting that opioids are found within projection neurons as well as within local circuit neurons.

In recent years, details of the connections of some opioid neurons have been established by combining lesion or tract tracing studies with immunohistochemical methods. In one of the first of these studies, it was found that enkephalin-immunoreactive cell bodies in the amygdala project to distant targets through the stria terminalis (Uhl et al. 1978). Prior to this work, many investigators felt that enkephalinergic neurons were restricted to participation in local circuits rather than projection pathways. Enkephalin- and dynorphin-immunoreactive neurons in the pontine and medullary raphe and reticular nuclei project to the spinal cord, and some of these fibers terminate in the vicinity of sympathoadrenal preganglionic neurons (Holets and Elde 1982; Appel et al., unpublished). Some of the opioidergic neurons in the striatum project to the substantia nigra (Zamir et al. 1984a). Opioid peptides are found in neurons of the superficial lamina of the dorsal horn of the spinal cord that are thought to be importantly involved in ascending, nociceptive pathways. Recently, a significant proportion of these opioidergic "marginal zone" neurons has been shown to project to the parabrachial nucleus of the upper pons (Standaert et al. 1986). Another ascending, putatively nociceptive pathway that produces opioid peptides arises from the vicinity of the central canal of the spinal cord (Nahin and Micevych, in press). These findings of opioids in neurons believed to respond to noxious stimuli may prompt development of significant new approaches for investigating basic aspects of pain and analgesia. Finally, enteric neurons that produce enkephalin immunoreactivity have been found to reside within the myenteric ganglia of the gut wall. These neuronal cell bodies send enkephalin-immunoreactive fibers orally to provide terminals in other myenteric ganglia, as well as

anally to supply the circular and muscular plexuses of the gut wall (Furness et al. 1983). Thus, the endogenous opioid neurons that regulate gut motility have been positively identified.

Apart from the numerous specific findings, some general principles have emerged. For the most part, if an individual neuron produces opioid peptides, all the opioid peptides produced appear to derive from a single biosynthetic precursor. Thus, neurons that produce ß-endorphin fail to produce either proenkephalin-derived peptides or prodynorphin-derived peptides (Watson et al. 1978). Similarly, the vast majority of neurons that produce either proenkephalin- or prodynorphin-derived peptides fail to produce peptides derived from other precursors of opioid peptides (Watson et al. 1982; Weber et al. 1982c; Khachaturian et al. 1985). However, two "exceptions" to this "rule" have recently been described in central neurons related to autonomic control. Guthrie and Basbaum (1984) described the coexistence of proenkephalin- and prodynorphin-derived peptides in some neurons within the nucleus of the solitary tract. We have described a similar coexistence in neurons of the dorsal gray commissure at the level of the sacral parasympathetic nucleus (Sasek and Elde, in press). The presence of two families of opioid peptides in individual neurons suggests that opioid production must be especially crucial for the signalling functions of such neurons.

Another general principle is that opioidergic neurons often produce additional, nonopioid transmitter substances, including acetyl choline (Altschuler et al. 1984), monoamines (Schultzberg et al. 1978; Glazer et al. 1981; Armstrong et al. 1984; Leger et al. 1986), GABA (Zahm et al. 1985), or other peptides (Hokfelt et al. 1980; Coulter et al. 1981; Erichsen et al. 1982; Weber et al. 1982c; Martin et al. 1983; Beauvillain et al. 1984; Morris et al. 1985). Most studies of the coexistence of neurotransmitters have focused on the neuronal cell body for the analysis. Recently, we have refined and characterized a two-fluorochrome, light microscopic method that allows studies of coexistence at the level of individual varicosities and nerve terminals (Wessendorf and Elde 1985). Preliminary studies in spinal cord suggest the existence of diverse combinations of putative transmitters in peptidergic nerve fibers and terminals (Appel et al. 1986; Appel et al., in preparation). Although the regional compartmentalization and combinations of chemical markers that coexist within opioid neurons are only beginning to be studied, we suspect that the unravelling of this chemical complexity will provide the basis for discerning functional divisions among opioidergic neurons. Such a vision is already realized, in part, for opioid neurons in the enteric nervous system. Furness and colleagues (1983) previously described the organization and connections of enkephalin-immunoreactive neurons in this preparation. In addition, they have characterized the electrophysiological properties of identified enteric enkephalinergic neurons (Bornstein et al. 1984)--a task that has been extremely difficult in mammalian central nervous system preparations. Their studies have now been expanded to include dynorphin and numerous other putative transmitter substances (Costa et al.

1985; Furness and Costa 1986). The expanded survey has demonstrated up to six discrete transmitter-related markers in individual neurons. Despite this myriad of neurochemical combinations, adding information about the connections and electrophysiological properties of individual neurons gives a unique, functionally meaningful accounting of those neurons. Given the power of this new level of knowledge about chemically coded neurons, it can be expected that significant advances in understanding the neurobiology of opioidergic neurons will arise from studies of this sort.

Species variations in the distribution of opioids have been noted (Elde et al. 1980), and it now appears that an enkephalinergic circuit in one species may be substituted by dynorphinergic neurons in another species. This is illustrated by the occurrence of prodynorphin-derived peptides in hypothalamic magnocellular neurons of the rat (Weber et al. 1982c) and proenkephalin-related peptides in the homologous neurons in the cat (Micevych and Elde 1980). Similarly, prodynorphin-derived peptides are abundant in the substantia nigra of the rat (Khachaturian et al. 1982; Khachaturian et al. 1985), whereas proenkephalin-derived peptides are more abundant in the homologous neurons of the nonhuman primate (Haber and Elde 1982). The production of one or another of the opioid precursor proteins can now be precisely determined by the differential localization of their respective messenger RNA by in situ hybridization. However, the identification of posttranslationally processed, releasable peptides continues to be an important task for immunocytochemical studies of nerve fibers and terminals.

LINGERING QUESTIONS CONCERNING THE NEUROBIOLOGY OF OPIOIDERGIC NEURONS

Although the regional and cellular distribution of opioidergic neurons is now fairly well understood, we know very little about the numerous neuronal circuits in which opioids appear to participate. In addition, several questions have emerged concerning the cellular mechanisms important in processing opioids. For many neurotransmitters, including the opioids, the sites of release remain to be clarified. Although it is expected that opioids and other transmitters are released at the terminal boutons of axons, it is unknown whether or not preterminal varicosities are also release sites. The axons of opioid neurons are generally unmyelinated and exhibit many varicosities along their course (figure 4). Unmyelinated, varicose axons with a similar morphology arise from postganglionic sympathetic neurons. Release of catecholamines and neuropeptides is suspected at the varicosities along the route of these axons. In contrast, axons of hypothalamic magnocellular neurons that terminate in the neural lobe of the pituitary exhibit striking varicosities (termed Herring bodies) which are not thought to be release sites for vasopressin and oxytocin. It is unknown whether or not other portions of the opioidergic neurons are capable of selective release of opioid peptides. For example,

Release Sites of Transmitters

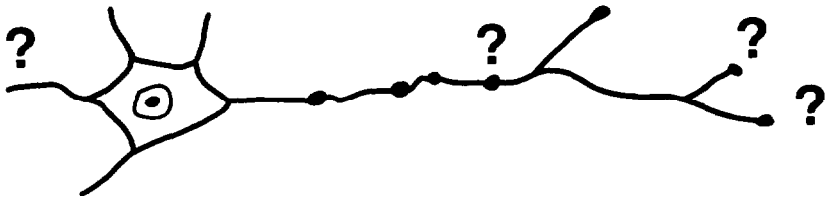

FIGURE 4. *Schematic illustration of a neuron, its dendrites, and an axon*

NOTE: The axon is illustrated to have varicosities along Its course. Similar varicosities represent sites for the release of neurotransmitters In the peripheral autonomic nervous system. It is not known whether or not varicosities along the course of opioidergic axons in the central nervous system represent potential sites of release.

it is suspected that dendrites of neurons in several brain regions may release transmitters to act "postsynaptically" on receptors on other dendrites (Shepherd 1979). Thus, the sites at which opioid peptides might be released from opioidergic neurons must be established in order to understand the sites where opioids might exert their action.

If release of opioid peptides occurs at varicosities along the course of an axon, it then becomes possible that the molecular structure of the opioid peptides may differ between proximal and distal release sites (figure 5). This possibility exists because axonal transport and posttranslational processing are both time-dependent phenomena. It has been found, for example, that the proximal axons of hypothalamic magnocellular neurosecretory neurons contain relatively unprocessed versions of preprovasopressin and preprooxytocin. More distally (i.e., with additional time), these axons contain increasing concentrations of the cleaved, biologically active neurohormones (Gainer et al. 1977; Brownstein et al. 1980). Thus, if opioid peptides are released from proximal axon collaterals or varicosities, one might expect that the profile of these peptides will be different from that of the peptides released at more distal terminals.

Releasable Forms of Peptides

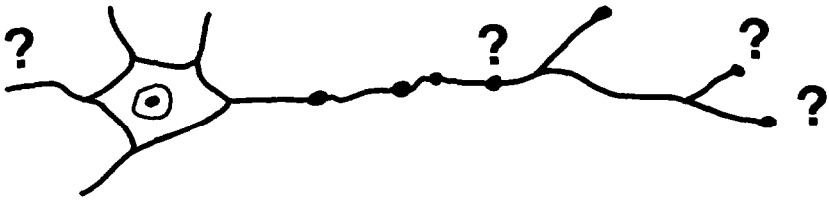

FIGURE 5. *Schematic illustration similar to figure 4*

NOTE: If preterminal varicosities might be sites for the release of neurotransmitters, It then becomes possible that the molecular forms of opioids and other peptides stored and available for release at proximal varicosities may be less processed than at the more distal terminals.

The relationship between release sites of opioid peptides and their sites of action also remains controversial. The somewhat parallel uncovering of multiple opioid peptides with different spectra of activity and multiple classes of opioid receptors has tempted many to propose a neurotransmitter-receptor linkage for a given opioid peptide type and opioid receptor class. Indeed, the semiselective binding properties of enkephalins (Chang et al. 1979), metorphamide (Weber et al. 1983), dynorphins (Chavkin et al. 1982; Corbett et al. 1982), and ß-endorphin (Akil et al. 1981; Akil et al. 1984) might be used to predict a parsimonious relationship between these endogenous ligands and particular types of binding sites. Unfortunately, the anatomical relationship between opioid peptides and opioid binding sites is not in any way straightforward (Lewis et al. 1984). Even the initial immunohistochemical studies of opioid peptides (Elde et al. 1976; Watson et al. 1978; Watson et al. 1981; Vincent et al. 1982; Weber et al. 1982b) failed to provide regional, rank order density ratings of these peptides that correlated well with regional, rank order density ratings of different classes of opioid binding sites (Atweh and Kuhar 1977; Chang et al. 1979; Goodman et al, 1980). We have also seen this discrepancy at the level of identified neurons within the intermediolateral cell column of the spinal cord. Although these neurons exist within a highly enriched plexus of enkephalin- (Holets and Elde 1982) and dynorphin-immunoreactive nerve fibers and terminals (Appel and Elde, unpublished), there is not a corresponding enrichment in dihydromorphine binding sites

(Seybold and Elde 1984). Current studies are seeking to determine whether or not other classes of opioid binding sites are enriched over these identified neurons. However, such mismatches between the density of binding sites and the abundance of opioidergic nerve fibers and terminals is a widely recognized phenomenon (Kuhar 1985; Herkenham and McLean 1986). Thus, an enigma is apparent in that potential release sites for opioids are, in many cases, separated from binding sites by significant biological distances. Among the more plausible explanations may be that autoradiographic binding studies typically reveal only high-affinity binding sites. It may be that low-affinity sites are more closely related to release sites for opioids, and that the high-affinity sites are occupied only during times of massive release of opioids that would lead to diffusion to these more distant sites (figure 6). This enigma is difficult to resolve with present techniques and may require the development of antibodies to discrete opioid receptor proteins which can then be employed for the immunohistochemical localization of all classes and affinities of opioid receptors. The resolution of this problem is extremely important for understanding the biology of opioid peptides and their receptors.

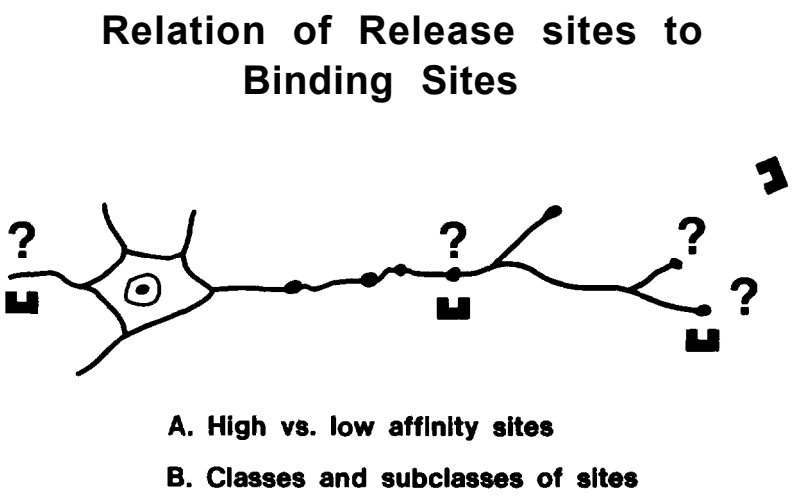

FIGURE 6. *Schematic illustration, similar to figures 4 and 5, to which neurotransmitter binding sites have been added*

NOTE: The details of the spatial ralationships between sites of release of opioid peptides and their binding sites are not known.

CONCLUSIONS

The complexity of neuronal relationships with respect to the production of the various opioid peptides and opioid receptors suggests that a great deal of effort will be required to solve the existing riddles. For the time being, it would seem prudent that efforts be directed at relatively simple opioidergic circuits and functions. One example of an exploitable model of opioidergic circuitry and many of its complexities is found in the enteric nervous system. Significant progress in clarifying the neurobiology of mammalian, central opioids will be made as techniques allow for the isolation and examination of the anatomy, chemistry, and physiology of individual neurons and their functional interactions.

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Opiate-Inhibited Adenylate Cyclase in Mammalian Brain Membranes

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INTRODUCTION

The identification of opiate receptor binding sites in brain membranes (Pert and Snyder 1973; Terenius 1973; Simon et al. 1973) demonstrated the existence of the first step in the molecular actions of opiates and endogenous opioid peptides. However, the second messenger of opioid systems has been more difficult to study. Although several potential opiate-coupled intracellular responses have been reported, the principal opiate-coupled second messenger system characterized in isolated membranes has been the inhibition of adenylate cyclase by opioid agonists. The first experiments showing opiate inhibition of cyclic AMP synthesis were conducted by Collier and Roy (1974), who showed that morphine inhibited adenylate cyclase in membranes from rat striatum. Although other studies of opiate-inhibited adenylate cyclase in brain membranes appeared at this time, they were limited, not only by the relatively small degree of inhibition by opiate agonists, but also by the extreme heterogeneities of brain membranes that produced small responses of adenylate cyclase to any known neurotransmitter (Drummond 1981). An easier system for the study of this reaction was the neuroblastoma-glioma hybrid cell line NG108-15, which contained opiate receptor binding sites (Klee and Nirenberg 1974) and represented a much less heterogeneous neural tissue than brain membranes. Several groups (Sharma et al. 1975; Traber et al. 1975) showed that morphine and enkephalin analogs inhibited adenylate cyclase in NG108-15 cells. Moreover, chronic exposure of these cells to morphine altered the adenylate cyclase response: basal adenylate cyclase activity was gradually increased during morphine exposure, while addition of naloxone caused a rebound increase in adenylate cyclase activity (Sharma et al. 1975). These results suggested that the development of opiate tolerance could be at least partially due to uncoupling of opiate receptors from adenylate cyclase. Since experiments had already shown that tolerant animals showed no changes in opiate receptor binding sites themselves (Pert and Snyder 1976), the findings with adenylate cyclase represented one of the first potential molecular mechanisms of tolerance.

At the same time, research in other areas determined the biochemical characteristics of hormone receptor-coupled adenylate cyclase (Rodbell 1980; Gilman 1984; Schramm and Selinger 1984). Receptors are coupled to the catalytic unit of adenylate cyclase through specific guanine nucleotide-binding (N) proteins. One, N_s, mediates stimulation of adenylate cyclase, while another, N_i, mediates inhibition (Gilman 1984). Each N protein is composed of three subunits, alpha, beta, and gamma. While the beta subunit is identical in both N_s and N_i, the alpha subunits are different, and function to bind and hydrolyze GTP. Thus, coupling of receptors with adenylate cyclase requires GTP. N proteins also perform a second function by interacting directly with receptor binding sites and allowing the formation of high-affinity agonist complexes; in the presence of GTP, the receptor/N protein complex dissociates and agonist affinity is decreased. The opiate receptor system operates in an analogous fashion. Opiate agonist binding sites are regulated by guanine nucleotides in both brain membranes (Blume 1978a; Childers and Snyder 1978; Childers and Snyder 1980) and membranes from NG108-15 cells (Blume 1978b). Moreover, opiate-inhibited adenylate cyclase in NG108-15 cells requires GTP (Blume et al. 1979).

It was important to extend the studies of opiate-inhibited adenylate cyclase in transformed cell lines to brain membranes, since the brain contains multiple binding sites not present in neuroblastoma cells (Chang and Cuatrecasas 1979). More recent studies on opiate-inhibited adenylate cyclase in brain membranes have become more consistent as the biochemical requirements of receptor-mediated adenylate cyclase become more clear. Thus, several studies (Cooper et al. 1982; Law et al. 1981) demonstrated the guanine nucleotide requirement for opiate-inhibited adenylate cyclase and determined some of the pharmacological properties of opiate-inhibited adenylate cyclase in brain membranes. Unfortunately, brain membranes remained a very heterogeneous system, and adenylate cyclase responsiveness was still not as good as that in the neuroblastoma-glioma cells.

To characterize the molecular properties of opiate receptor coupling to adenylate cyclase, our studies focused on techniques that modified the interactions of N proteins with receptors and adenylate cyclase. One such technique involved low pH pretreatment of brain membranes to alter coupling of opiate receptors with adenylate cyclase. This report summarizes our recent findings in brain membranes altered by low pH pretreatment. This treatment allows the quantification of opiate-inhibited adenylate cyclase and the determination of receptor subtypes involved in coupling with adenylate cyclase.

METHODS

Opiate-Inhibited Adenylate Cyclase

For determination of low pH effects on adenylate cyclase, membranes were resuspended in 0.5 ml of either adenylate cyclase

buffer, pH 7.4, or low pH buffer (50 mM sodium acetate, 5 mM $MgCl_2$, 1 mM dithiothreitol, pH 4.5), and incubated at 0 °C for 20 minutes. The low pH reaction was terminated by addition of 4 ml cyclase buffer; the membranes were centrifuged at 30,000 x gravity for 10 minutes and resuspended in cyclase buffer. For assay of adenylate cyclase, membranes (10-50 µg protein per tube) were added to tubes containing 20 mM creatine phosphate, 10 units creatine phosphokinase, 10 mM theophylline. 700 µg bovine serum albumin, and 100 µM (1 $_{\mu}$CI) ^{3}H-ATP in a total of 100 µl. For assay of opiate-inhibited activity, the tubes contained 100 mM NaCl and 50 mM GTP in addition to the appropriate drugs. The adenylate cyclase reaction was initiated by addition of ATP, the tubes were incubated for 10 minutes at 30 °C, and the reaction was terminated by boiling for 2 minutes. Separation of the labelled cyclic AMP was performed on a reverse-phase high performance liquid chromatography (HPLC) column. This technique was modified (Childers 1986) from the method of Schulz and Mailman (1984), using ^{3}H-ATP as substrate and adenosine deaminase to reduce levels of adenosine, which interfere in the HPLC peak of cyclic AMP.

Chronic Drug Treatments

Rats were treated with naltrexone according to the method of Zukin et al. (1982). Naltrexone (775 mg/ml, dissolved in saline) was placed in Alzet osmotic mini-pumps (Model 2001, 1 µl/hr), which were implanted subcutaneously (SC) in male Sprague-Dawley rats (200 to 250 gm). Rats were treated for 7 days, then sacrificed. Forebrains were assayed for ^{3}H-D-Ala enkephalinamide (D-Ala enk) binding, and striatal membranes were assayed for opiate-inhibited adenylate cyclase after low pH pretreatment as described above.

For chronic morphine studies, rats were implanted SC with one 75-mg morphine (free base) pellet, then 2 days later with two more pellets, then sacrificed 3 days after the second implantation. Some morphine-tolerant rats were injected intraperitoneally (IP) with 1 mg/kg naloxone and sacrificed 1 hour later. For acute morphine studies, 10 mg/kg morphine was injected IP, then rats were sacrificed 20 to 60 minutes later. Striatal membranes were assayed for opiate-inhibited adenylate cyclase as described above.

Miscellaneous Assays

Opiate receptor binding assays were conducted as previously described (Childers and Snyder 1978), using H-D-Ala enk (40.6 Ci/mmole) or H-naloxone (56.4 Ci/mmole) as labelled ligands. For experiments using p-nitro-phenyl-oxymorphone (PNPO), membranes were incubated with 10 µM PNPO at 25 °C for 30 minutes, then washed twice by centrifugatfon before assay of receptor binding and adenylate cyclase. To ensure removal of the reversible component of PNPO binding, control membranes were incubated in parallel with 10 µM oxymorphone; after washing was completed, these membranes exhibited binding identical to untreated control membranes (Childers 1984). In experiments with phospholipase-A, membranes were incubated with 0.5 µg of the enzyme with 1 mM $CaC1_2$

for 20 minutes at 25 °C. The reaction was terminated by addition of 5 mM EGTA, and membranes were assayed for receptor binding and adenylate cyclase. Protein values were determined by the method of Lowry et al. (1951).

RESULTS AND DISCUSSION

Effects of Lou pH Pretreatment on Brain Adenylate Cyclase

When brain membranes were preincubated in sodium acetate buf er at pH 4.5 prior to assay of opiate receptor binding at pH 7.7, ^{3}H-opiate agonist binding was unaffected, while the maximum inhibition of agonist binding by guanine nucleotides was increased approximately twofold (Childers and Jackson 1984; Lambert and Childers 1984). Since these results suggested that low pH pretreatment altered interactions of N proteins with receptors in brain membranes, we tested whether the same pretreatment would alter N protein interactions with adenylate cyclase. Results (Childers and LaRiviere 1984) showed that while low pH pretreatment did not change basal adenylate cyclase activity, it decreased stimulation of adenylate cyclase by agents that act through N_s; these agents included fluoride, stable guanine nucleotides such as Gpp(NH)p, and dopamine, which stimulates adenylate cyclase through D_1 receptors.

Measurement of opiate-inhibited adenylate cyclase has always been difficult to quantitate in brain membranes because of the small effect of opiate agonists in this tissue. Recent literature has reported maximum inhibition of 15 to 25 percent, depending on the study (Cooper et al. 1982; Law et al. 1981). In our experiments, we were unable to demonstrate reproducible opiate inhibition of adenylate cyclase of more than 10 to 15 percent. However, when brain membranes were preincubated at pH 4.5 before assay of adenylate cyclase at pH 7.4, the decrease in N_s-stimulated adenylate cyclase resulted in an increase in opiate-inhibited adenylate cyclase to a maximum of 30 to 40 percent inhibition. The inhibition of adenylate cyclase by D-Ala enk was mediated by opiate receptors, since the dose response curve of the agonist was shifted to the right by naloxone (figure 1).

This experiment also showed that D-Ala enk inhibited activity at µM concentrations. While such concentrations may seem high compared to affinities of D-Ala enk at receptor binding sites in Tris buffer, they are reasonable concentrations when used in physiological buffers containing sodium and guanine nucleotides. In fact, when binding studies were conducted in adenylate cyclase buffer, under conditions identical to those required to demonstrate opiate-inhibited adenylate cyclase, D-Ala enk displaced ^{3}H-naloxone binding at µM concentrations (not shown). Even conducting binding assays in adenylate cyclase buffer, with GTP but without sodium, decreased both agonist and antagonist binding (table 1). Under these conditions, the binding of the mu compounds ^{3}H-DAGO and ^{3}H-naloxone was most affected, with greater than 70 percent inhibition in adenylate cyclase buffer compared to Tris buffer

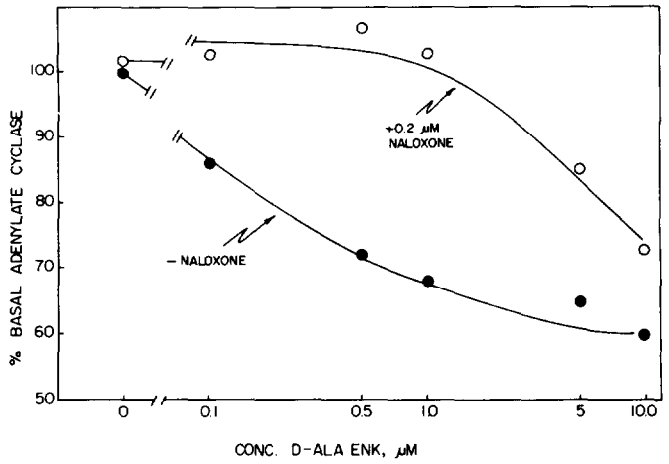

FIGURE 1. *Inhibition of adenylate cyclase in low pH pretreated striatal membranes by D-Ala enk in the absence and presence of 0.2 μM naloxone*

NOTE: Maximum inhibition in nontreated membranes was approximately 10 percent.

containing 50 μM GTP. The mixed specificity ligands D-Ala enk and EKC were inhibited by approximately 60 percent, while diprenorphine and DSLET were less affected (20 to 30 percent inhibition). This decreased binding of both agonists and antagonists in adenylate cyclase buffer is caused by the addition of theophylline to prevent cyclic AMP hydrolysis in the adenylate cyclase assay, and demonstrates that high-affinity (nM) binding sites should not be expected under conditions that maximize receptor coupling to adenylate cyclase.

TABLE 1. *Opiate receptor binding in Tris buffer and adenylate cyclase buffer*

	Specific cpm Bound		
^{3}H-Ligand	Tris Buffer	Cyclase Buffer	% Bound
^{3}H-D-Ala enk	6,744	2,744	41
^{3}H-DAGO	5,181	1,195	23
^{3}H-DSLET	5,480	3,894	71
^{3}H-EKC	4,010	1,594	40
^{3}H-naloxone	4,895	1,224	25
^{3}H-diprenorphine	3,089	2,528	82

Mechanism of the Low pH Effect

To explore the mechanism of low pH-induced changes in receptor/N protein/adenylate cyclase interactions in brain membranes, several different possibilities were explored. One of these possibilities was that low pH pretreatment changed membrane structure to alter the interactions of membrane proteins. Changes in membrane fluidity alter beta-adrenergic receptor interactions with adenylate cyclase (Hanski et al. 1979), so we explored this possibility by adding membrane fluidizing agents to low pH-pretreated brain membranes (Childers and LaRiviere 1984). Results (not shown) demonstrated that addition of lipids like phosphatidyl choline or cis-vaccenic acid had no effect on adenylate cyclase in untreated membranes, but reversed the effects of low pH pretreatment. Thus, fluoride-stimulated adenylate cyclase was restored, while opiate-inhibited adenylate cyclase was reduced. In fact, a significant inverse correlation was discovered between N_s-stimulated and opiate-inhibited adenylate cyclase: as N_s-stimulated activity was reduced, opiate-inhibited activity was increased. Interestingly, this reversal effect of lipids was not observed for opiate receptor binding sites: in low pH-pretreated membranes, the guanine nucleotide regulation of agonist binding was increased, whether lipids were added or not (Childers and LaRiviere 1984).

These results suggest that a low pH-induced change in lipid structure of brain membranes may alter N protein interactions with receptors and adenylate cyclase. The difference between reversals of low pH effects on receptors and adenylate cyclase might suggest that these two functions of GTP are mediated through different N proteins, or, alternatively, that interaction of an N protein with either receptors or adenylate cyclase may be regulated by different factors in membranes. Therefore, predictions cannot be made concerning efficiency of receptor/adenylate cyclase coupling from the degree of regulation of agonist binding by guanine nucleotides.

Properties of Opiate-Inhibited Adenylate Cyclase in Brain Membranes

The effect of low pH pretreatment to increase opiate-inhibited adenylate cyclase in brain membranes to a maximum of 30 to 40 percent inhibition allowed the quantitative examination of this activity in brain.

Lineweaver-Burke plot of enzyme kinetics experiments (figure 2) showed that inhibition by 10 µM D-Ala enk was noncompetitive, decreasing the V_{max} but not the Km of adenylate cyclase for ATP. This class of inhibition is typical for other neurotransmitter-inhibited activities (Drummond 1981).

Opiate-inhibited adenylate cyclase required at least 1 µM GTP, and 50 µM GTP was required for full inhibition (not shown). Gpp(NH)p, which stimulated basal activity by 50 percent, and forskolin,

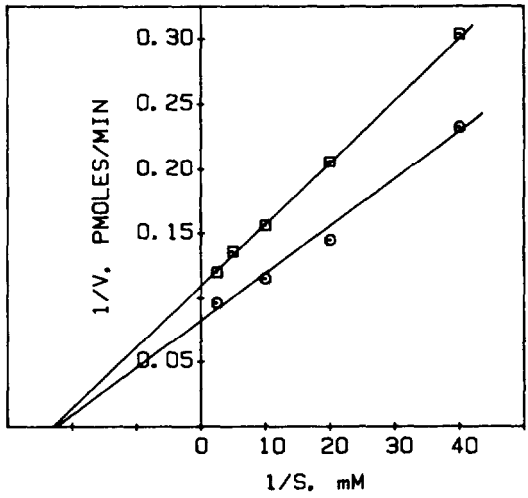

FIGURE 2. *Lineweaver-Burke kinetics of basal (circles) and D-Ala enk-inhibited (squares) adenylate cyclase in low pH pretreated striatal membranes*

which stimulated activity threefold, did not support any significant inhibition by D-Ala enk (not shown).

Table 2 shows the agonist specificity of opiate-inhibited adenylate cyclase in low pH-pretreated striatal membranes. Most opioid peptides exhibited μM affinities, with maximal inhibitions between 30 and 35 percent. Enkephalin, enkephalin analogs, beta-endorphin, and dynorphin were all effective agonists in this system. N-acetyl-beta-endorphin, which has very little affinity for opiate receptors, was ineffective in inhibiting adenylate cyclase. Moreover, morphine and the mu opioid peptides were weaker and less effective than the other opioid peptides in inhibiting activity. Therefore, mu receptors are probably not involved in opiate receptor-inhibited adenylate cyclase.

The actions of antagonists in blocking D-Ala enk-inhibited adenylate cyclase in striatal membranes are summarized in tables 3 and 4. To determine the antagonist specificity of D-Ala enk-inhibited adenylate cyclase in low pH-pretreated membranes, a series of opioid and nonopioid antagonists were added at 1-μM concentrations to block 10 μM D-Ala enk-inhibited adenylate cyclase (table 3). All nonopioid antagonists were completely ineffective in blocking D-Ala enk-inhibited activity. Opioid antagonists such as naloxone,

naltrexone, and levallorphan totally blocked inhibition at 1-μM concentrations. Interestingly, the delta antagonist ICI-174864 did not block D-Ala enk inhibition at 1-μM concentrations; increasing the concentration of ICI-174864 to 10 μM was only slightly more effective in blocking D-Ala enk inhibition (not shown).

TABLE 2. *Specificity of opiate-inhibited adenylate cyclase in rat striatal membranes*

Opioid	IC_{50}, μM	Maximal Inhibition	
		% Inhibition	Concentration, μM
Met-enk	0.8	33	10
Leu-enk	0.6	30	10
D-Ala enk-NH_2	0.8	32	10
DADL enk	0.7	32	10
DSLET	0.3	32	10
Beta-end	0.8	40	10
N-Ac beta end		11	50
Dyn (1-13)	0.7	30	10
Dyn A (1-17)	0.6	31	10
D-Ala dyn A	0.6	40	10
Morphine	6	18	100
DAGO	10	24	50
Morphiceptin	10	15	50

TABLE 3. *Effect of antagonists on striatal opiate-inhibited adenylate cyclase*

Neurotransmitter	Antagonist	% Inhibition
None	None	35±4
Muscarinic ACh	Atropine	30±4
Dopamine	Chlorpromazine	38±6
Beta-adrenergic	Propranolol	32±7
5-HT, dopamine	Spiroperidol	39±3
5-HT, histamine	Mianserin	39±2
$Alpha_2$-adrenergic	Yohimbine	32±6
Opioid	Naloxone	6±5
Opioid	Naltrexone	2±6
Opioid	Levallorphan	8±4
Dpioid	ICI-174864	36±6

K_e values for naloxone were determined using 100 to 500 nM naloxone to shift dose response curves of several agonists (table 4). Naloxone was effective in blocking the actions of all the agonists tested, and the K_e values of naloxone against the delta peptide DADL-enkephalin, the mu peptide FK-33824, and beta-endorphin were approximately equal, ranging from 50 to 80 nM. Again, although these K_e values of naloxone appear high compared to receptor binding data, the values of 50 to 80 nM are not very different from K_e values of naloxone in blocking delta receptors in mouse vas deferens (Kosterlitz and Paterson 1980). On the other hand, naloxone was considerably weaker against dynorphin, with a K_e of 400 nM.

TABLE 4. *Striatal opiate-inhibited adenylate cyclase: K_e values of naloxone*

Agonist	Naloxone Ke, nM
D-Ala, D-leu enkephalin	86±25
FK-33824	77±26
Beta-endorphin	68±31
Dynorphin-A	441±129

The distribution of opiate-inhibited adenylate cyclase in several regions of rat brain is seen in table 5. Of all the regions tested, only striatum and amygdala provided good (at least 30 percent) inhibition of adenylate cyclase. Thalamus exhibited moderate inhibition, and hypothalamus contained moderate to low inhibition, but all other regions had no real, significant opiate-inhibited adenylate cyclase. This distribution does not follow the distribution of any known opiate receptor binding sites in rat brain.

Effects of Chronic Drug Treatments on Opiate-Inhibited Adenylate Cyclase

Zukin et al. (1982) demonstrated that chronic treatment of rats with naltrexone increased numbers of opiate receptors in brain membranes. Moreover, these increased sites were more sensitive to regulation by guanine nucleotides, suggesting that receptor/N protein coupling might be affected by chronic antagonist treatment. To test this latter hypothesis, we treated rats for 1 week with naltrexone and assayed opiate receptor binding in forebrain membranes and opiate-inhibited adenylate cyclase in low pH-pretreated striatal membranes. Results (table 6) showed that chronic treatment with naltrexone did significantly increase receptor binding by an average of 60 percent, similar to results reported by Zukin et al (1982). However, we did not observe any change in regulation of 'H-D-Ala enk binding by GTP. Moreover, in the treated animals, there was no change in opiate-inhibited adenylate

cyclase in either the affinity of D-Ala enk or the maximal inhibition. Therefore, the increase in receptor binding sites is not accompanied by a change in receptorladenylate cyclase coupling, as predicted from changes in GTP regulation of binding (Zukin et al. 1982). This confirms, as mentioned above, the danger in predicting the degree of receptor/adenylate cyclase coupling based on data of guanine nucleotide regulation of binding.

TABLE 5. *Regional distribution of opiate-inhibited adenylate cyclase in rat brain membranes*

Region	Basal Activity, pmoles/min/mg/	Maximal % Inhibition*	
		+DSLET	+DAGO
Striatum	63.4	32	29
Amygdala	32.1	37	31
Thalamus	37.4	22	20
Sensomotor cortex	13.8	15	11
Hypothalamus	53.4	10	11
Hippocampus	12.6	9	12
Frontal cortex	10.7	5	0
Brain stem	11.3	5	1
Cerebellum	34.4	4	1

*Assayed in the presence of 10 μM OSLET and 50 μM DAGO, together with 100 mM NaCl, 50 μM GTP, and crude rat brain membranes.

TABLE 6. *Effect of chronic naltrexone treatment on opiate receptor binding and adenylate cyclase*

Treatment	% Specific Binding		Adenylate Cyclase	
	-GTP	+GTP	D-Ala enk IC_{50}	Max. % Inhib.
Control	100±12	39.9±2.1	5.522±3 μM	29.1±7.9
Naltrexone	159±16	40.0±2.3	6.8±3.7 μM	25.5±6.1

NOTE: Rats were treated with 75 μg/hr naltrexone for 7 days, then striatal membranes were assayed for H-D-Ala enk binding ± 50 μM GTP and for inhibition of adenylate cyclase by 1 to 50 μM D-Ala enk.

To determine the effects of chronic morphine treatment on opiate-inhibited adenylate cyclase, rats were implanted SC with morphine pellets for 1 week and assayed for opiate-inhibited adenylate cyclase in low pH-pretreated striatal membranes. Results (table 7) showed that neither acute injection of morphine (10 mg/kg

injected 20 minutes before sacrifice), chronic morphine treatment, nor naloxone withdrawal following chronic morphine treatment significantly altered inhibition of adenylate cyclase by D-Ala enk.

TABLE 7. *Effect of chronic morphine treatment on D-Ala enk-inhibited adenylate cyctase*

Treatment	Adenylate Cyclase Activity		
	% Basal	IC_{50}, μM	Max. % Inhibition
Control	100±12	10.1±1.3	20.8±1.7
Acute morphine	112±17	10.2±2.0	14.0±1.8
Chronic morphine	113±10	16.1±6.5	31.4±3.4
Naloxone withdrawal	98±9	9.0±1.3	18.7±3.8

These results suggest that the development of morphine tolerance does not involve a change in the coupling between receptors and adenylate cyclase; however, morphine is not a good agonist in the opiate-inhibited adenylate cyclase system, and therefore is not the agonist of choice in chronic drug treatments. We are currently exploring this possibility by chronic treatment of rats with a better agonist in this system, D-Ala enk.

Receptor Subtypes Coupled to Adenylate Cyclase in Brain

The pharmacological properties of opiate-inhibited adenylate cyclase in brain membranes demonstrated, as discussed above, that mu receptors were not coupled to adenylate cyclase in brain, thus leaving delta receptors as prime candidates for the receptor subtype coupled to adenylate cyclase. However, although delta opioid peptides were relatively potent in inhibiting adenylate cyclase, and although K_e values for naloxone were similar to those at delta receptors, several lines of evidence made this conclusion untenable. First, other nondelta opioid peptides were as potent as delta opioid peptides, including beta-endorphin and dynorphin. Second, the regional distribution of opiate-inhibited adenylate cyclase does not follow that of delta binding sites. Finally, although classical mu antagonists such as naloxone and naltrexone were effective in blocking opiate-inhibited adenylate cyclase, the delta-specific antagonist ICI-174864 was ineffective up to 10-μM concentrations.

To explore the relationship between opiate binding sites and the receptor-coupled adenylate cyclase, we utilized agents that block high-affinity opiate receptor binding. Naloxone azine, an irreversible antagonist at mu(1) sites (Hahn et al. 1982), was an effective antagonist in blocking opiate-inhibited adenylate

cyclase, but this blo kade was totally washed out by centrifugation steps that kept ^{3}H-naloxone binding inhibited by 75 percent (data not shown). An even more effective irreversible compound is p-nitro-phenyl-oxymorphone (PNPO), an agonist that blocks opiate receptor binding sites at nM concentrations (Pasternak, submitted for publication). When PNPO was added to low pH-pretreated striatal membranes, it inhibited adenylate cyclase in a manner similar to that of morphine, with an affinity of 10 μM and maximal inhibition of 25 percent (not shown). Irreversibility of PNPO at both receptor binding sites and with adenylate cyclase is demonstrated in table 8. In these experiments, 10 μM PNPO was incubated with striatal membranes, then the reversible component was washed out by centrifugation. PNPO could not be washed away from receptor binding sites, as seen by persistent 80 to 99 percent inhibition of ^{3}H-DSLET, ^{3}H-DAGO, and ^{3}H-EKC binding. Surprisingly, even after this significant inhibition of binding, D-Ala enk continued to inhibit adenylate cyclase, with maximal inhibition identical to that in nontreated membranes.

TABLE 8. *Effect of p-nitro-phayl-oxymorphole on opiate receptor binding and opiate-inhibited denylate cyclase in striatal membranes*

A. Receptor Binding

	Control		Treated	
Ligand	Specific cpm	% Control	Specific cpm	% Control
^{3}H-DAGO	3,166	100	27	1
^{3}H-DSLET	3,169	100	619	20
^{3}H-EKC	1,572	100	235	15

B. Adenylate Cyclase

	% Basal Activity	
Additions	Control	Treated
None	100	101
D-Ala enk-NH_2	61	65
Naloxone + D-Ala enk-NH_2	94	96

Even more dramatic separation of binding and opiate inhibition of adenylate cyclase was demonstrated with phospholipase-A, an enzyme known to inhibit opiate receptors at low concentrations (Pasternak and Snyder 1975), but which does not inhibit basal adenylate cyclase (Anderson et al. 1978; Partington and Daly 1979; Lad et al. 1979). When low pH-pretreated striatal membranes were incubated

with 0.5 µg/ml phospholipase-A, receptor binding was eliminated, whether assayed with ^{3}H-DSLET, ^{3}H-DAGO, or ^{3}H-EKC (table 9). However, opiate-inhibited adenylate cyclase was not affected by this treatment: although basal adenylate cyclase activity was slightly increased, activity was still inhibited by more than 30 percent by D-Ala enk. Thus, several different techniques reveal that opiate-inhibited adenylate cyclase persists in membranes in which mu, delta, and kappa agonist binding has been eliminated.

TABLE 9. *Effect of phospholipase A on opiate receptor binding and opiate-inhibited adenylate cyclase in striatal membranes*

A. Receptor Binding

	Control		Treated	
Ligand	Specific cpm	% Control	Specific cpm	% Control
^{3}H-DAGO	3,166	100	91	3
^{3}H-DSLET	3,169	100	12	0
^{3}H-EKC	1,572	100	109	7

B. Adenylate Cyclase

	% Basal Activity	
Additions	Control	Treated
None	100	118(100)
D-Ala enk-NH_2	71	88 (74)
Naloxone + D-Ala enk-NH_2	97	117 (99)

These results strongly suggest that the receptor coupled to adenylate cyclase in brain is neither mu, delta, nor kappa sites as measured by classical binding methods. This conclusion should not be too surprising, since the conditions used to maximize receptor/adenylate cyclase coupling are totally different from those used to maximize high-affinity receptor binding. In fact, as stated earlier, high-affinity (nM) agonist receptor binding is not detectable in buffers containing sodium and GTP. Moreover, there is precedence in the study of cell systems to show that classical opioid binding sites are not coupled to adenylate cyclase. For example, when 95 percent of delta binding sites were blocked by chlornaltrexamine in NG108-15 cells, opiate-inhibited adenylate cyclase was unaffected (Fantozzi et al. 1981). In addition, detailed pharmacological experiments questioned the delta identity of receptor-coupled adenylate cyclase in these same cells (Costa et al. 1985). Finally, in C-6 glioma cells, where opiate receptors and opioid inhibition of cyclic AMP levels were induced by

treatment of cells with desmethylimipramine (Tocque et al. 1984), the receptor binding sites were not delta sites, suggesting that opiate-inhibited adenylate cyclase in this system was not mediated by delta receptors. This evidence, coupled with the results reported here, strongly suggests that receptors coupled to adenylate cyclase represent a novel class of receptor sites not described so far in binding studies. This receptor has μM affinity for opioid peptides, is blocked by 50 to 100 nM concentrations of naloxone and naltrexone, and is present primarily in striatum and amygdala. Despite the fact that this new receptor class is not one of the classical binding sites, it is nevertheless a genuine opiate receptor, since it exhibits agonist specificity (for example, N-acetyl beta-endorphin is much less effective than beta-endorphin itself) as well as antagonist specificity (only opiate antagonists block the opioid peptide inhibition). Whether this class of receptor is the same as that seen in cells such as C-6 glioma or NG108-15 is not yet clear.

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Membrane Conductances and Opioid Receptor Subtypes

R. Alan North

INTRODUCTION

There is a great deal of information available regarding the actions of opioids on individual nerve cells (Duggan and North 1984). The principal direct action is a reduction in the rate of firing of action potentials; this has been observed in numerous brain regions. A second action that also occurs at many sites is a depression of the amount of transmitter released at nerve terminals (presynaptic inhibition). In the last few years, it has become possible to study the primary effects of opioids that underlie these two actions at the level of the cell membrane. This has been made possible by a combination of two techniques. The first is an old one. Pharmacological null methods (Gaddum 1937; Schild 1947) have been applied to the study of single nerve cells, and these have allowed the measurement of agonist and antagonist dissociation equilibrium constants (K_es). This is important because opioids can interact with more than one kind of receptor subtype, and the least ambiguous way to determine the subtype is to measure the antagonist K_e. The null methods provide a measure of K_e which is independent of the coupling between receptor and effect. The second technique was more recently developed. Membrane currents can be measured from individual neurones in either the central or peripheral nervous system, maintained *in vitro*. It is important to study single cells, because not all neurones, even in a given brain nucleus, respond equally to opioids. Voltage-clamp methods are needed, because the membrane-bound ion channels that are affected by opioids may also be affected by membrane potential; potential must therefore be controlled. *In vitro* study of the cells is essential so that concentrations of opioids and ions can be carefully controlled. This is readily done in a tissue bath.

EFFECTS OF μ RECEPTOR ACTIVATION

There is now abundant evidence for the presence of opioid receptor subtypes. These are generally classified on the basis of antagonist K_es, either in ligand binding studies or in pharmacological

experiments on isolated tissues. The μ receptor is defined for present purposes as one that has a naloxone K_e of 0.5 to 3 nM, is activated by selective agonists such as morphiceptin (Tyr-Pro-Phe-Pro-NH_2) in the low nanomolar concentration range, and is irreversibly blocked by ß-funaltrexamine (ß-FNA) (Goldstein and James 1984). In rat locus coeruleus cells (Williams and North 1984) (antagonist affinities, ß-FNA), and on mouse dorsal root (MDR) ganglion cells (Werz and Macdonald 1983a) (ß-FNA, selective agonists), μ receptor activation increases a potassium conductance. In the locus coeruleus, this hyperpolarizes the cell and inhibits action potential discharge. A similar action has been observed in other neurones including rat substantia gelatinosa (Yoshimura and North 1983), myenteric neurones (Morita and North 1982), and hippocampal interneurones (Nicoll and Madison 1984). The underlying receptor has not been carefully defined in these experiments, but the effectiveness of morphine as an agonist implicates the μ receptor (see below). In the hippocampus, extracellular recording of field synaptic potentials indicates that they are enhanced by opioids acting on μ receptors (Bostock et al. 1984). Since the increase of the field potential results from an inhibition of interneurones (Nicoll and Madison 1984), it is likely that the interneurones themselves have μ receptors.

The potassium conductance increased by μ receptor activation in the rat locus coeruleus has been described in some detail (North and Williams 1985). The maximal conductance increase caused by opioids is about 5 nS, and the value is independent of membrane voltage between -120 and -50 mV. The conductance is also not sensitive to 4-aminopyridine (1 mM) (which reduces the A-current in the same cells by about 70 percent); nor is it sensitive to tetraethylammonium (10 mM) or apamin (up to 1 μM). The conductance is significantly reduced by barium (100 to 300 μM) and by quinine (100 μM) and is completely blocked when barium is used to replace extracellular calcium (usually 2.5 mM). These results allow one to "fingerprint" the particular conductance or set of channels affected by opioids and to distinguish it from other potassium conductances in the same or other cells.

Although the conductance increase by opioids becomes smaller at membrane potentials less negative than about -50 mV, it still contributes to action potential repolarization. Thus, opioids acting on μ receptors shorten the action potential in locus coeruleus neurones by increasing the potassium conductance; their effect on the action potential is lost when the action potential is recorded in conditions of substantial potassium channel blockade (intracellular caesium or extracellular barium) (North and Williams 1983). This action of opioids serves to modulate calcium entry indirectly (Kandel 1981); if such an action occurred at transmitter release sites, it would cause presynaptic inhibition of release. Such <u>indirect</u> modulation of calcium entry (that is, by an increase in potassium conductance), as a means of reducing transmitter release, contrasts with <u>direct</u> modulation; in direct modulation, the activated receptor reduces the calcium conductance

without any change in potassium conductance (see discussion of κ receptors below).

In dorsal root ganglion cells, opioids act on μ receptors to increase a potassium conductance that contributes to the action potential repolarization and also to the action potential after hyperpolarization (Werz and Macdonald 1983a; Werz and Macdonald 1983b). This conductance has been studied by measuring the duration of the action potential prolonged by tetraethylammonium. As in the locus coeruleus, opioids that are selective for μ receptors do not shorten the action potential which has been prolonged by intracellular caesium. It is concluded that the potassium conductance which is increased by opioids in the dorsal root ganglion cells has slightly different properties from that affected in the locus coeruleus. It is not known whether this might be accounted for by the difference in species or the immaturity of the dorsal root ganglion cells.

EFFECTS OF δ RECEPTOR ACTIVATION

The existence of the δ receptor as a separate entity on single neurones has also been shown by experiments of the kind described above. δ receptors are coupled to a potassium conductance in cells of the guinea pig submucous plexus (Mihara and North, in press). The properties of this potassium conductance are very similar to that increased by μ opioids on locus coeruleus or other cells (see above). The naloxone K_e for antagonism of [Met]enkephalin hyperpolarizations is different from that found in locus coeruleus neurones, and ICI174864 (N-bisally-Tyr-Aib-Aib-Phe-Leu) (Aib = aminoisobutyrate), a δ receptor antagonist (Cotton *et* al. 1984), acts as a competitive antagonist in submucous neurones with a K_e of about 30 nM. This is in sharp contrast to the results in the locus coeruleus, where ICI174864 also acts as an antagonist of [Met]enkephalin, but the K_e is more than 10 μM (Williams and North 1984). The agonist potencies on submucous plexus neurones are also strongly indicative of the δ receptor type. Morphine, FK33824 (Tyr-D-Ala-Gly-MePhe-Met(O)-ol), and DAGO (Tyr-D-Ala-Gly-MePhe-Gly-ol), which are typical μ receptor agonists, and dynorphin A and tifluadom, which are κ receptor agonists, do not affect potassium conductance at concentrations up to 1 μM. The selectivity displayed by these agonists for opioid receptors on locus coeruleus (μ) and submucous plexus (δ) cells is actually considerably greater than that seen in ligand binding studies using prototypic ligands (DAGO and Tyr-D-Pen-Gly-Phe-D-Pen (DPDPE)), suggesting perhaps that the preparation of membranes for ligand binding experiments may result in a loss of agonist selectivity. On the other hand, there is good quantitative agreement between antagonist K_es measured on single neurones and in binding studies on brain homogenates.

Mouse DRG cells also have δ receptors, because selective δ agonists such as Tyr-D-Pen-Gly-Phe-Pen (DPLPE) cause spike shortening. Morphiceptin is inactive in many of the neurones affected by DPLPE, suggesting the existence of only δ and not μ receptors in

some cells (see below). The δ agonists have no effect after blockade of potassium conductance, indicating that, like μ agonists. they indirectly modulate the entry of-calcium ions by-shortening the duration of the action potential (Werz and Macdonald 1983a; Werz and Macdonald 1983b).

EFFECTS OF k RECEPTOR ACTIVATION

The κ agonists dynorphin, tifluadom, and U50,488H have no effect on neurones of the rat locus coeruleus (μ receptors) (Williams and North 1984) or guinea pig submucous plexus (δ receptors) (Mihara and North 1986) in concentrations up to 1 μM. However, some guinea pig myenteric neurones are affected by κ agonists. The effect observed is a shortening of the action potential, but this differs from the findings with μ and δ receptor agonists, because it still occurs after the action potential has been prolonged by intracellular caesium. This indicates that the κ agonists directly modulate calcium entry into the neurone by an action that does not involve potassium channels.

Direct suppression of calcium currents by κ agonists was first suggested by Werz and Macdonald (Werz and Macdonald 1984a; Werz and Macdonald 1984b; Werz and Macdonald 1985). They recorded membrane currents from voltage-clamped mouse dorsal root ganglion cells and found that these were inhibited by κ opioids but not by μ or δ selective agonists. There are other reports that opioids directly depress calcium conductance (Mudge et al. 1979; Bixby and Spitzer 1983), but in those experiments the receptor subtype was not known, Because relatively high concentrations of agonists were used, it is possible that the effects resulted from activation Of a κ receptor.

The significance of this action of opioids on transmitter release can be assessed by measuring the amplitude of the nicotinic fast excitatory postsynaptic potential (epsp) in the myenteric plexus. This can be taken as a measure of the amount of acetylcholine released by a single action potential in a few presynaptic fibers. Superfusion of either μ or κ agonists depresses the epsp by acting on the terminals of presynaptic fibers. These fibers have their cell bodies within the myenteric plexus, and it is therefore reasonable to suppose that the depression by the agonists (see above) of the calcium action potential observed in the cell bodies might also be occurring near the terminals; this could be the reason for the presynaptic inhibition. In keeping with this notion is the finding that barium, which prevents the increase in potassium conductance, blocks the presynaptic inhibitory action of morphine but not that of dynorphin (Cherubini and North 1985).

DO DIFFERENT OPIOID RECEPTORS EXIST ON THE SAME CELL?

Some neurones of the myenteric plexus show effects of both morphine (hyperpolarization) and dynorphin (reduction in calcium spike). Similarly, many of the epsps in the myenteric plexus are

depressed by both morphine and dynorphin (Cherubini and North 1984). Although the receptors involved have not been characterized by K_e determinations, the agonists were used at concentrations at which they were likely to be selective. This indicates that μ and κ receptors probably coexist on the same myenteric neurones.

Mouse DRG neurones seem to fall into four groups (Werz and Macdonald 1985; Werz and Macdonald, personal commnunication). The first group (about 50 percent of cells) shows no effects of opioids, which may be because they do not express any of the receptor types. The second group (about 25 percent) shows effects of κ agonists only--that is, the action potential is shortened even when recording with a caesium-filled electrode. The third group exhibits effects of both κ and μ agonists, and the fourth group, kκ and δ. In view of the finding that the effects of activating μ and δ receptor types seem to be identical, it is of some interest that individual neurones seem not to express both μ and δ receptors. One would question the evolutionary value of conserving two opioid receptors which both open the same kind of potassium channel in the membrane of a single cell if they respond only to one naturally occurring ligand; it is more likely that they would represent minor variations of a single gene product. Conversely, if neurones are found which possess both μ and δ receptors, one might hypothesize that there exists a more highly selective natural ligand for the μ receptors than any of those presently known.

OPIOIDS AND NONOPIOIDS HAVE SIMILAR ACTIONS

Noradrenaline has the same effect as opioids in neurones of the rat locus coeruleus (Williams et al. 1985), the guinea pig myenteric plexus (Surprenant and North 1985), and the guinea pig submucous plexus (North and Surprenant 1985). In the rat locus coeruleus and the guinea pig submucous plexus, the receptors that underlie this action of noradrenaline have been characterized by measurement of antagonist K_es. They belong to the α_2 class. There is no difference between the properties of the potassium conductance increased by opioids and that increased by α_2 -adrenoceptor agonists on the same cell, with respect to sensitivity to barium, quinine, or rubidium, or insensitivity to 4-aminopyridine and tetraethylammonium. Furthermore, the maximum conductance increase that can be evoked by an agonist acting on the opioid receptor (μ in the case of the locus coeruleus. and δ in the case of the submucous plexus) is the same as that which can be evoked by an agonist acting at the α_2 receptor on that cell. When the current responses to agonists of each type are added, it is found that the same population of potassium channels is opened by both (Williams and North 1984; Mihara and North 1986). In the guinea pig submucous plexus, somatostatin also increases the same potassium conductance by acting on a receptor that is neither δ nor α_2 (not blocked by naloxone or idazoxan).

It is less clear whether precisely the same calcium conductance is depressed by κ agonists as that affected by other substances. However, depression of calcium conductance is an effect of several different neurotransmitters (for example, γ-aminobutyric acid, adenosine, and catecholamines). In some cases, κ opioids inhibit calcium entry in the same neurone as one or more of these substances, and since there are more discrete receptors than types of calcium channels, it seems likely that the different receptor mechanisms will also converge prior to the effect on the channel.

CONCLUSIONS

The principal actions of opioids on the properties of mammalian neurones can be ascribed to an inhibition of the rate of discharge of action potentials and to a reduction in the amount of transmitter released by each action potential passing along the axon. Opioids of the μ and δ types achieve both these actions by opening membrane ion channels that allow outward movement of potassium ions and thereby hyperpolarize the membrane; the outward potassium current also serves slightly to reduce the duration of the action potential, and this seems to be responsible for the presynaptic inhibition of transmitter release. These opioid actions are shared by α_2-adrenoceptor agonists (and somatostatin) acting on their own receptors; all the cells examined so far appear to express both an opioid (μ or δ) and an α_2 receptor. Opioid receptors of the κ class mediate inhibition by a quite different cellular mechanism; they directly depress calcium conductance. This contributes to presynaptic inhibition of transmitter release, and it may also inhibit the firing of cells that are normally spontaneously active as a result of inward calcium currents at normal membrane potentials.

The elucidation of the ionic conductances which are coupled to the three opioid receptor subtypes allows comparisons to be made with receptor subtypes for other transmitters. Individual transmitters such as γ-aminobutyric acid, acetylcholine, and noradrenaline each act on a variety of receptor subtypes; in each case, one subtype inhibits a calcium conductance and another increases a potassium conductance. In one sense, therefore, the opioids are conventional. In another sense, they are very different in that the naturally occurring ligands for at least two of the receptor subtypes (μ and κ) are encoded on different genes; dynorphin and [Met]enkephalin might be considered to have little in common except the same N terminal amino acids.

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Modulation of Opioid Peptide Metabolism by Seizures: Differentiation of Opioid Subclasses

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INTRODUCTION

Evidence is accumulating that the proenkephalin and prodynorphin families of opioid peptides play fundamentally different roles in the regulation of brain excitability, particularly in the seizure-sensitive hippocampus. Electrophysiological studies have indicated that leu^5- and met^5-enkephalin have a net excitatory action when iontophoretically applied to pyramidal cells in the hippocampus, whereas in most other brain areas these peptides manifest inhibitory actions (Nicoll et al. 1977). Intracellular recordings from pyramidal cells in the CA1 (or superior) region of the hippocampus have indicated that enkephalin-induced excitation, which is naloxone-reversible, may be due to inhibition of interneurons (Zieglgansberger et al. 1979); however, this interpretation is controversial (Corrigall 1983). In contrast, iontophoretically applied dynorphin peptides produce both naloxone-reversible excitatory effects and non-naloxone-reversible inhibitory effects in the hippocampus (Henriksen et al. 1982; Walker et al. 1982; Gruol et al. 1983; Moises and Walker 1985), indicating that more than one receptor subtype in the hippocampus may be affected by dynorphin peptides. Furthermore, intracerebroventricular (ICV) application of enkephalins and ß-endorphin have naloxone-reversible seizurogenic properties (Urca et al. 1977; Henriksen et al. 1978), whereas ICY dynorphin (Henriksen et al. 1982) and several synthetic opioid agonists directed against both mu and delta receptors (Tortella et al. 1984; Tortella et al. 1985) do not. Thus, opioid agonists may have proconvulsant or anticonvulsant effects depending on the dose and class of opioid receptors that they activate (Frenk 1983).

The demonstration of convulsant and anticonvulsant properties of opioid agonists has suggested that one endogenous opioid class may mediate seizure activity, while another may mediate the electrographic refractory state and behavioral depression that follow a seizure (postictal state). An involvement of opioids in the postictal state is supported by evidence that naloxone pretreatment diminishes the behavioral postictal depression that follows

seizures elicited by amygdaloid kindling (Frenk et al. 1979) or by electroconvulsive shock (Holaday and Belenky 1980; Tortella and Cowan 1982b; Tortella et al. 1985; Urca et al. 1981). If different opioid peptides are involved in seizure occurrence and postictal depression, then one would expect that recurrent seizures would have a different effect on enkephalin and dynorphin systems in seizure-sensitive brain areas. This hypothesis is strengthened by demonstrations that electroconvulsive shock treatments (Hong et al. 1979; Hong et al. 1985b; Kanamatsu et al., in press), kainic acid administration (Hong et al. 1980; McGinty et al. 1983; Obie et al. 1985; Kanamatsu et al., in press), and amygdaloid kindling (McGinty et al., in press) differentially alter enkephalin and dynorphin peptide levels in the hippocampus, reflecting changes in opioid metabolism in response to seizures. The data, reviewed herein, suggest that experimentally induced epileptiform activity may be a useful model in the study of the control of opioid metabolism in general.

LOCALIZATION OF HIPPOCAMPAL OPIOID PEPTIDES

The initial approach was to identify the pathways in the hippocampal formation that contain opioid peptides. Research has shown that two of the three major excitatory circuits of the hippocampal formation, the lateral perforant/temperoammonic (PTA) pathway and the dentate granule cell-mossy fiber (MF) pathway, contain opioid peptides. Proenkephalin is present in the PTA pathway (Gall et al. 1981; McGinty et al. 1982; McGinty et al. 1983; McGinty et al. 1984), which is the first link in a trisynaptic excitatory chain from the entorhinal cortex to the dentate granule cells and pyramidal cells of the hippocampus (figure 1, A and B). Prodynorphin and proenkephalin are contained in the granule cell-mossy fiber path (McGinty et al. 1983), which is the second link in the trisynaptic chain. Within the mossy fiber system of normal rats, however, the concentration of prodynorphin is much greater than the concentration of proenkephalin (figure 1, C and O). Recently, studies have shown that different populations of granule cells and hippocampal interneurons contain dynorphin B and bovine adrenal medullary peptide (BAM 22P), a proenkephalin-derived peptide (McGinty et al. 1984; McGinty et al. 1985; figure 1, E). Thus, in addition to scattered opioid interneurons, the hippocampus contains one pathway (PTA) that contains only proenkephalin and one pathway (MF) that contains proenkephalin and prodynorphin peptides in different neurons.

The distribution of opioid peptides partially overlaps the distribution of opioid receptors in the hippocampal formation. In vitro autoradiography demonstrates that ligands that predominantly bind mu, kappa, and lambda receptors are most densely distributed in the CA3-4 pyramidal cell layer and the dentate molecular layer (Meibach and Maayani 1980; Duka et al. 1981; McLean et al. 1985; Perry and Sadee 1985; Chang et al., in press). Delta-selective binding overlaps the distribution of mu and kappa sites, but it is

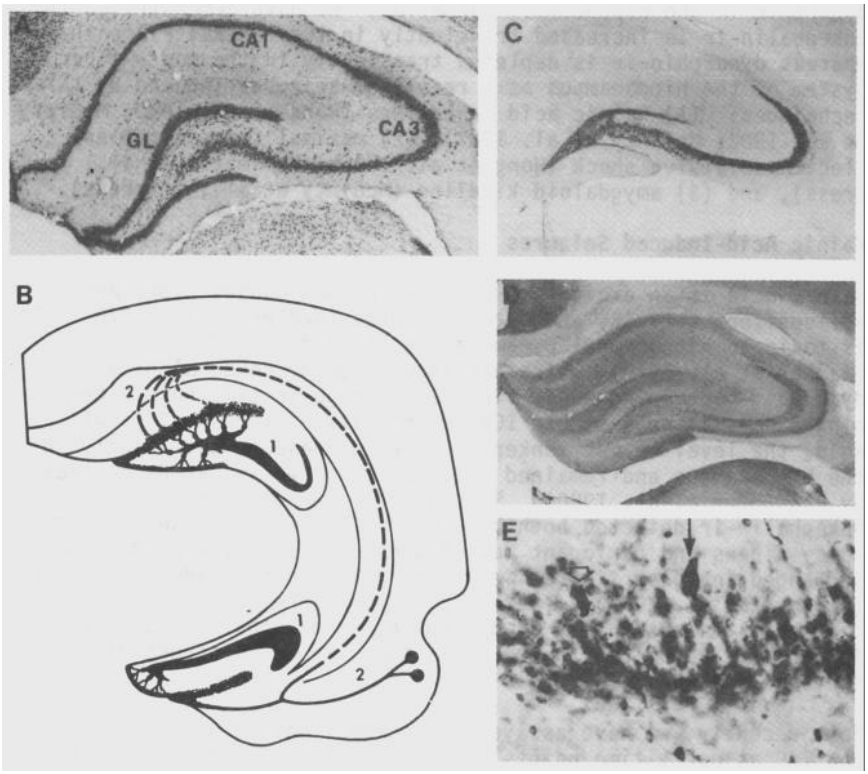

FIGURE 1. *Localization of hippocampal opioid peptides*

NOTE: A. Nissi-stalned coronal section of the dorsal hippocampal formation showing the dentate granule cell layer (GL) and pyramidal cell layers of CA3 and CA1 (x6.8). B. Schematic illustration of the major dynorphin-ir and enkephalin-ir hippocampal pathways (McGinty et al. 1983). (1) Dynorphin/enkephalin-ir mossy fibers originating from granule cells In both the dorsal and ventral dentate gyrus. (2) Enkephalin-ir lateral perforant/temperoammonic path originating In the lateral entorhinal cortex and innervating the outer molecular layer of the dentate gyrus and hippocampal fields CA1-CA4. Enkephalin-ir is densest in the caudal-ventral divisions of the entorhinal pathway. C. Dynorphin-ir in the mossy fibers of the dorsal hippocampus In a normal Sprague-Dawley rat (x4.3). D. Leu^5-enkephalin-ir In mossy fibers of a normal Sprague-Dawley rat (x4.8). E. Dentate granule cells In the ventral hippocampal formation stained with a two-color immunoperoxidase method (McGinty 1985). Right cell (arrow) contains dynorphin B-ir, and left cell (open arrowhead) contains BAM 22P-ir (x533).

lighter in the pyramidal and granule cell layers and more extensive in other layers of the hippocampal formation (McLean et al. 1985; Chang et al., in press).

DIFFERENTIAL RESPONSES OF OPIOID PEPTIDES TO SEIZURES

Our evidence from radioimmunoassay (RIA) and immunocytochemical (ICC) measurements of opioid peptides subsequent to seizures is consistent with the concept of different actions of enkephalin and dynorphin in the hippocampal formation. We have demonstrated that enkephalin-ir is increased transiently in the lateral PTA pathway, whereas dynorphin-ir is depleted transiently in the mossy fiber system of the hippocampus as a result of seizures induced by three techniques: (1) kainic acid injections (Hong et al. 1980; McGinty et al. 1982; McGinty et al. 1983), (2) maximal transauricular electroconvulsive shock (Hong et al. 1985b; Kanamatsu et al., in press), and (3) amygdaloid kindling (McGinty et al., in press).

Kainic Acid-Induced Seizures

Kainic acid is an excitatory amino acid that causes hippocampal epileptiform activity and motor seizures (Nadler et al. 1978). It is toxic to hippocampal pyramidal cells in fields CA3 and CA4 (Nadler et al. 1978), which are innervated by enkephalin- and dynorphin-containing mossy fibers. At regular time points beginning 2 days after a single ICV or intrastriatal dose of kainic acid, the level of met^5-enkephalin-ir increased significantly in the hippocampus and remained elevated for 2 weeks, as demonstrated by RIA (Hong et al. 1980). Subsequently, we have found that leu^5-enkephalin-ir detected both by RIA and ICC was increased in the mossy fibers and perforant path fibers, whereas dynorphin-ir was unchanged in mossy fibers 4 days after kainic acid administration (McGinty et al. 1983). These alterations were the first indication that the metabolism of dynorphin and enkephalin could be differentially regulated, not only in the same brain structure but in the same pathway.

Most recently, we have assayed hippocampal opioid peptides by RIA and ICC at early time points after a single intrastriatal injection of kainic acid (Obie et al. 1985; Kanamatsu et al., in press) (table 1). Recurrent seizures were still apparent 3 to 6 hours after kainic acid administration; dynorphin (1-8)-ir in mossy fibers was depleted 70 percent, and met-enkephalin-ir in the mossy fibers and perforant path was depleted by 30 to 40 percent. Within 24 to 48 hours, the levels of both peptides had risen above normal, with dynorphin-ir peaking at 48 hours at 171 percent of normal and enkephalin-ir, at 270 percent of normal. However, by 72 hours, enkephalin had continued to rise to 300 percent of normal, especially in the mossy fibers, while dynorphin-ir in the mossy fibers had returned to normal levels. The reduction in dynorphin-ir and enkephalin-ir within 3 to 6 hours of kainic acid administration suggests an increase in the release of these peptides during the seizure period. The rebound in peptide levels in later phases may be due to a transient overproduction of dynorphin and a

TABLE 1. *Hippocampal opioid peptide metabolism in different seizure models*

Seizure Model	Dynorphin A	Met^5-Enkephalin
Kainic Acid		
6 hr	-70%	-30%
24 hr	+35%	+95%
48 hr	+65%	+170%
72 hr	+0%	+200%
2 wk		+60%*
ECS		
1 day after: 1 ECS	+10%	+0%
3 ECS	-30%	+0%
6-10 ECS	-70%	+40%
2 wks after: 6-10 ECS	+0%	+0%
Kindling		
1 day after:		
stage 3 (subconvulsive)	+0%	+0%
stage 5 (convulsive)	-70%	-50%
28 days after stage 5	+0%	+0%
Seizure-Sensitive Gerbil		
Postseizure interval:		
72 hr	+34%	+24%
5 min	+34%	+100%

*Ipsilateral to intrahippocampal injection.

larger, more sustained overproduction of enkephalin. This possibility is supported by the demonstration of a sharp increase in proenkephalin messenger RNA in the hippocampus, as measured with a cDNA clone for proenkephalin (Hong et al. 1985b; Kanamatsu et al., in press) which peaked at 6 to 12 hours and returned to normal levels by 48 hours after kainic acid administration. These dramatic biphasic changes, the details of which are distinct for dynorphin-ir and enkephalin-ir, demonstrate the need to take neurochemical measurements at frequent intervals after seizure induction in order to detect a pattern of rapid changes and even reversals in peptide levels and mRNA. Only by constructing a

continuous time course of changes during and after seizure activity were we able to detect how dynamic the regulation of opioid peptide biosynthesis is.

Electroconvulsive Shock

Electroconvulsive shock (ECS) is the second seizure model that we have begun to explore in relation to the metabolic plasticity of opioid peptides (table 1). Several lines of evidence suggest that opioid peptides may be involved in ECS-elicited behavioral alterations such as analgesia, retrograde amnesia, changes in seizure threshold, and postictal depression (Urca et al. 1981; Urca et al. 1983; Holaday and Belenky 1980; Carrasco et al. 1982; Tortella and Cowan 1982b; Berman and Adler 1984). Twenty-four hours after 6 to 10 daily maximal ECS treatments (150 V at 60 Hz for 1-second duration), the levels of dynorphin (1-8)-ir and met^5-enkephalin-ir in several limbic-basal ganglia brain regions and the level of mRNA coding for preproenkephalin in the hypothalamus were significantly increased. In the hippocampal formation, however, met^5-enkephalin-ir increased by 40 percent in the perforant pathway, whereas dynorphin-ir decreased by 70 percent in the mossy fiber system in the absence of any cellular damage (Hong et al. 1985a; Kanamatsu et al., in press). These alterations in opioid peptide levels returned to normal by 2 weeks posttreatment. Repeated ECS treatments also downregulated mu and delta opioid receptor binding in the hippocampus, hypothalamus, and caudate nucleus, but not in the frontal cortex or brain stem (Nakata et al. 1985). Scatchard analysis demonstrated that the number, not the affinity, of opioid sites was reduced for up to 2 weeks after the last of 10 daily ECS treatments. The fact that both enkephalin and dynorphin systems are affected in many brain regions after ECS is consistent with a general involvement of these peptides in ECS-elicited behavioral alterations. However, the opposite changes in hippocampal enkephalin and dynorphin levels after ECS suggest that these two peptides must play fundamentally different roles, possibly by interacting with different receptor subtypes, in mediating hippocampal excitability.

Amygdaloid Kindling

In order to see if postseizure changes in opioid peptides were a common response to seizure induction in the hippocampal formation, we employed yet another model of epilepsy. Amygdaloid kindling is an experimental model of temporal lobe epilepsy in which initially subthreshold intra-amygdaloid stimulations result in progressively more intense seizure activity (stages 1 to 4) until a generalized (stage 5) seizure occurs (Goddard 1967; Racine 1972). More work has been focused on discovering the neurochemical correlates of kindling than on any other seizure model; despite this effort, there is no prevailing consensus about whether defects in specific neurotransmitters or their receptors are responsible for the kindling phenomenon (McNamara 1984).

Several lines of evidence have suggested a relationship between endogenous opioid peptides and 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-ir has been reported to be increased in whole rat brain (Vindrola et al. 1981), and dynorphin-ir has been observed to increase 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 1). One day after stage 3 seizures, no significant change in opioid peptide levels was observed by RIA or ICC, indicating that opioid peptides are not involved in the process of kindling before the stage of generalized seizures. In contrast, 1 day after two cons cutive stage 5 seizures, there was a 50 percent increase in met^5-enkephalin-ir in the temperoamnonic pathway and a 70 percent decrease in dynorphin (1-8)-ir in the mossy fibers of the hippocampus (McGinty et al., in press). The only other change was an increase in the immunostaining of dynorphin in the substantia nigra, pars reticulata, a structure recently 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 in the absence of any apparent seizure activity. Since all of the above neurochemical changes after kindling appear to be transient in nature, the permanence of the kindling phenomenon cannot be explained by these alterations.

SEIZURE-SENSITIVE GERBILS

In contrast to models of epilepsy that depend on seizure induction by an exogenous trigger, there are strains of rodents that have spontaneous seizures. Immediately after a gerbil has a spontaneous seizure, we have demonstrated recently that both enkephalin and dynorphin levels of the hippocampus are significantly elevated compared to those levels in seizure-resistant gerbils (Lee et al., in press) (table 1). However, the elevation in enkephalin occurs only after a seizure, whereas the elevation in dynorphin levels is present in seizure-sensitive gerbils regardless of when a seizure occurs. It has also been reported that seizure-sensitive gerbils have an increased number of GABA-immunostained basket cells in the dentate gyrus of the hippocampus (Peterson et al. 1985). This increase in GABA-containing structures is in contrast to a reduced number of GABA-immunostained terminals in cortical epileptic foci (Ribak et al. 1979). Electron microscopic evidence has demonstrated, however, that the GABA-immunostained basket cells in the dentate gyrus of the seizure-sensitive gerbil innervate (i.e., inhibit) each other instead of inhibiting the granule cells as they would in normal gerbils (Peterson and Ribak 1985). Therefore, one should expect fundamental differences between the patterns of neurochemical changes in seizure induction models and

genetic models of epilepsy, perhaps reflecting a similar diversity in the human epilepsies.

SEPTOHIPPOCAMPAL DIFFERENTIATION

Brain cholinergic, monoaminergic, and GABA systems are the most widely implicated in seizure transmission and inhibition (Woodbury 1984). All of these neurotransmitter systems are present in neurons intrinsic to or afferent to the hippocampal formation and have been implicated in the regulation of hippocampal excitability. Of these, we have preliminary evidence that destruction of the primarily cholinergic septohippocampal pathway leads to alterations in endogenous opioid metabolism in the absence of seizures. Four days after fornix transections (Hong et al. 1981) or lesions of the medial septal nuclei (McGinty et al. 1985), enkephalin-ir was increased in the temperoammonic pathway, but no change was observed in dynorphin-ir in the mossy fibers. These preliminary data indicate that dynorphin and enkephalin metabolism in these two hippocampal pathways are differentially regulated. The data also indicate that perturbations of hippocampal pathways that do not induce seizures, but may alter seizure sensitivity to other stimuli such as kindling (Gellman and McNamara 1984), can alter opioid peptide metabolism in the hippocampal formation.

SUMMARY AND CONCLUSIONS

Until now, we have measured dynorphin-ir and enkephalin-ir at only a few time points after a single seizure or after multiple seizures in most of the models we have employed. Except for the genetically seizure-prone gerbil, our data consistently show a transient and robust decrease in dynorphin-ir and a sustained increase in enkephalin-ir in the hippocampal formation subsequent to kainic acid-, ECS- , or amygdaloid-kindled convulsive seizures. At this point, kainic acid appears to have the most dramatic effects on hippocampal enkephalin and dynorphin levels, causing an initial decrease followed by a rebound increase beyond control levels, which, for met^5-enkephalin, is maintained for at least 2 weeks. Recurrent seizures leading to neurotoxic effects on CA3 pyramidal cells, which are not present after ECS or kindling, may underlie the sustained alteration in enkephalin metabolism after kainic acid. Further investigation into the time course of seizure-induced enkephalin and dynorphin metabolic changes using RIA, ICC, and measurements of opioid mRNA levels may reveal a common pattern of depletion due to immediate release, rebound synthesis according to the severity of demand, and stabilization at a new equilibrium over several days or even weeks in each seizure model.

Our preliminary time points suggest striking differences in the rate of metabolism of hippocampal dynorphin and enkephalin in response to seizures. We would like to find out if other perturbations of the hippocampus, primarily the elimination of the influence of its known neurochemical afferents by lesion (as performed on the septohippocampal system described above) or pharmacological blockade, can alter the metabolism of hippocampal opioid

peptides and influence subsequent seizure transmission. Distinguishing the physiological conditions that induce metabolic changes in discrete opioid peptidergic pathways may help us to understand how endogenous opioids are involved in the regulation of neuronal excitability in specific brain regions, as well as to understand more about the differential regulation of opioid peptide metabolism in different brain pathways.

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Kappa Isoreceptors: Neuroendocrine and Neurochemical Evidence

Paul L. Wood and Smriti Iyengar

INTRODUCTION

The concept of a major opioid receptor triad consisting of mu, delta, and kappa receptors (Wood 1982) is now well established. The wide diversity of behavioral and pharmacological effects of opiate analgesics and opioid peptides has been a key argument for the existence of multiple opioid receptor systems. The establishment of reliable binding assays has further aided in the pharmacological and biochemical delineation of these receptor differences (Wood 1983a). In the case of the mu receptor population, there has been a further subclassification into mu-1 and mu-2 isoreceptors (Wood 1984; Pasternak 1982).

MU-1 Systems

- Analgesia induced by drug injections directly into the periaqueductal gray (PAG) is effective with mu and delta agonists but not with kappa agonists (Wood et al. 1983).
- Cortical and hippocampal acetylcholine (ACh) turnover is suppressed by mu and delta agonists but not by kappa agonists (Wood et al. 1984b; Wood 1984).
- Catalepsy is induced in the rat by mu, delta, and epsilon agonists but not by kappa agonists (Pasternak 1982).

MU-2 Systems

- Striatal dopamine metabolism is accelerated by mu and delta agonists but not by kappa agonists (Wood et al. 1980; Wood 1983b; Wood 1984; Wood et al. 1984b).
- Respiratory depression is induced by mu and delta agonists but not by kappa agonists (Wood et al. 1982; Ling et al. 1983).

- In studies of physical dependence, it has become evident that many of the signs of precipitated withdrawal in rats are mu-2 and delta mediated (Ling et al. 1983). Withdrawal signs with kappa agonists are different from those with a mu or delta agonist and are much less severe (Woods et al. 1982).

In the above systems, the isoreceptor populations were defined via inhibition or lack of inhibition by the selective mu-1 antagonist naloxonazine (Hahn et al. 1982; Wood and Pasternak 1983), or by the mu-2 antagonist actions of presently available kappa agonists (Wood 1984).

KAPPA ISORECEPTORS IN THE CENTRAL NERVOUS SYSTEM

The examples given do not represent all the available evidence for mu isoreceptor populations, but were listed to demonstrate a key point of interest in early studies with kappa agonists. Specifically, a large number of systems appeared that were lacking kappa receptor regulation; it was not until more physiological assays specific for kappa agonists appeared that the concept of kappa isoreceptors could be evaluated.

- The rabbit vas deferens is a kappa-selective peripheral tissue (Oka et al. 1981).

- The guinea pig esophagus appears to be most sensitive to kappa agonists (Kamikawa and Shimo 1983).

- In the dog mesenteric artery, kappa agonists appear to selectively depress norepinephrine release and elicit dilatation (Sun and Zhang 1985).

- Spinal analgesia and inhibition of substance P-induced scratching in the rat were potently elicited by kappa agonists (Wood et al. 1981; Rackham et al. 1981), presumably via kappa receptors located in the dorsal horn of the spinal cord (Gouarderes et al. 1985).

- The endogenous kappa agonist dynorphin$_{1-8}$ is contained within vasopressin neurosecretory vesicles in the rat neurointermediate lobe (Whitnall et al. 1983; Zamir et al. 1984) along with specific kappa receptors on pituicytes (Bunn et al. 1985). This system is presumably involved in the diuretic actions of kappa agonists (Leander 1983).

Binding Studies

Initial binding studies with membrane homogenates also were able to demonstrate a unique kappa binding site, which was characterized by low affinities for mu, delta, and epsilon agonists but high affinity for kappa agonists (Wood 1982); greater resistance to proteolytic agents (Pasternak 1980); greater resistance to alkylation (Wood and Charleson 1982); and greater resistance to heat inactivation (Wood and Pilapil 1983). These studies led to

the ultimate evidence for receptor heterogeneity: solubilization of the kappa receptor (Itzhak et al. 1984).

Receptor heterogeneity among the published kappa binding sites became evident when multiple cold displacers were coevaluated within one laboratory. This was the case with analysis of ^{3}H-SKF-10,047 binding in rat brain (Pilapil and Wood 1983), ^{3}H-etorphine binding in guinea pig spinal cord (Attali et al. 1982), ^{3}H-etorphine binding in guinea pig striatum in the presence of mu and delta receptor blockers (Audigier et al. 1982), and ^{3}H-EKC binding in guinea pig brain in the presence of mu and delta receptor blockers (Su 1985). These studies all consistently described two kappa receptor sites; both sites possessed high affinities for the synthetic kappa agonists, but one site possessed preferential affinities for dynorphin$_{1-17}$, and the other site, in contrast to previously described kappa sites, possessed high affinities for met-enkephalin Arg^{6}Phe^{7}, beta-endorphin, DADLE, and morphine. Both sites were unaffected by PCP, suggesting kappa isoreceptor populations and no PCP receptor component; however, a sigma receptor involvement has not been excluded.

These binding studies were the first to suggest the potential of kappa isoreceptors in the central nervous system (CNS). Subsequent in vivo validation of this concept has been slower in momentum, with the first data coming from detailed studies of kappa regulation of the hypothalamic-pituitary-adrenocortical (HPA) axis in the rat (Iyengar et al. 1985).

Corticosterone Release

Studies of the actions of a series of kappa and agonist/antagonist analgesics on corticosterone release in the rat have clearly demonstrated a kappa regulation of this neuroendocrine system. The key characteristics of these actions (Iyengar et al. 1985) are as follows:

- stereospecific increases in serum corticosterone;
- stereospecific reversal by opiate antagonists;
- dose dependency with a rank order of drug potency similar to that observed in the rabbit vas deferens (i.e., a kappa-specific tissue);
- no cross tolerance with mu agonists;
- tolerance in subchronic studies;
- inactivity in hypophysectomized rats; and
- no sigma receptor role involved in the actions of kappa agonists.

These data argue for a potent kappa regulation of the HPA axis and suggest an action at the level of the hypothalamus or pituitary with regard to corticosterone release. In further studies of the antagonism of these actions of kappa agonists by the narcotic antagonists naloxone and WIN44,441, we observed that while the actions of tifluadom and EKC are blocked by both of these antagonists, the actions of MR-2,034 and U50,488H were only reversed by naloxone (table 1). WIN44,441 was inactive up to 5 mg/kg, a dose that is efficient in blocking the actions of mu agonists in this system. Similarly, WIN44,441 was active in blocking morphine-dependent analgesia and increases in striatal dopamine metabolism (Wood et al. 1984a).

TABLE 1. *Summary of actions of kappa agonists a plasma corticosterone, plasma TSH, DOPAC levels in the olfactory tubercle, and cerebral arteries in vitro*

Parameter	MR-2,034	U50,488H	Tifluadom	EKC
Corticosterone				
% control (mg/kg, IP)	800(4)	600(8)	600(2)	700(8)
Naloxone reversal	Y	Y	Y	Y
WIN44,441 reversal	N	N	Y	Y
TSH				
% Control (mg/kg, IP)	25(16)	26(8)	50(10)	20(16)
Naloxone reversal	Y	Y	Y	Y
WIN44,441 reversal	N	N	Y	NT
DOPAC (Olf. Tub.)	149(4)	150(16)	IA(16)	IA(16)
Naloxone Reversa!	Y	Y	-	-
WIN44,441 Reversal	N	N	-	-
Cerebral Arteries*	Cont	Cont	NT	Relax

NOTES: Y = yes; N = no, up to 5 mg/kg IP; NT = not tested; IA = inactive; Cont = contraction; Relax = relaxation.

*Altura et al. (1984).

TSH Release

Preliminary studies of kappa regulation of TSH release in the rat (Iyengar and Wood 1985) have also delineated a stereospecific kappa-dependent decrease in plasma TSH. As with corticosterone, these actions express no cross-tolerance with morphine and a differential antagonism by narcotic antagonists (table 1).

DOPAC in Olfactory Tubercle

Previous studies have documented the regulation of the nigrostriatal and ventral tegmental-nucleus accumbens dopamine projections by mu and delta agonists, but not by kappa agonists (Wood 1983a). In fact, the mu-2 and delta antagonist actions of presently available kappa agonists have been demonstrated with regard to these pathways (Wood 1983a; Wood 1984). In contrast, it appears that the dopamine input to the olfactory tubercle of the rat is regulated by mu, delta, and kappa opioid systems. The actions of MR-2,034 and U50,488H were naloxone--but not WIN44,441--reversible, while tifluadom and EKC were inactive on this dopaminergic pathway up to 16 mg/kg (table 1).

SUMMARY

It has become clear, in the area of opiate research, that there are multiple opioid receptors and endogenous ligands. The functional roles of these opioid systems are being established as these concepts evolve and as our analytical capabilities become more sophisticated and thorough. When complete dose-response studies are performed and a number of antagonists are examined, a clearer definition of isoreceptor populations may also occur. Such appears to be the case with kappa agonists, which may in fact comprise two groups of compounds (table 1). This separation into two groups has also been observed in the dog cerebral artery (table 1) (Altura et al. 1984). Taken together, these data are sufficient to propose the testable hypothesis that there are kappa isoreceptor populations in the brain that probably subserve a vast array of discrete and different regulatory functions.

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Multiple Receptor Types in Opioid Discrimination

Stephen G. Holtzman

INTRODUCTION

Drugs can serve as interoceptive stimuli to govern behavioral responses in much the same way as exteroceptive environmental stimuli, such as auditory or visual cues, can govern behavior. In most discrimination paradigms designed to study the interoceptive stimulus properties of drugs, an animal is trained to emit one response after being injected with a reference drug and to emit a different response after being injected with the drug vehicle. Responses have included turning to the left or right in a T-maze escape procedure or, more commonly, activating one of two manipulanda in order to receive appetitive reinforcement or to avoid or escape from presentation of an aversive stimulus. A well-trained subject will reliably emit the response appropriate for the drug condition not only when injected with the reference drug used for training but also when injected with a novel drug that has pharmacologic properties similar to those of the training drug. Thus, stimulus control of behavior is generalized from the training drug to the novel drug, presumably because the novel drug and the training drug give rise to similar interoceptive stimuli. On the other hand, subjects usually emit the response appropriate for the vehicle condition when tested with a novel compound that is dissimilar pharmacologically from the training drug.

The exquisite pharmacologic specificity of this behavioral paradigm lends itself well to systematic investigation of mechanisms of action of drugs from a variety of classes. There is perhaps no better example of this than the application of drug discrimination methodology to the study of opioid drugs. That opioid agonists and antagonists interact with several distinct but stereochemically similar receptors was suggested by the differences in the profiles of action of opioid drugs in a variety of *in vivo* and *in vitro* assay systems (Martin et al. 1976; Gilbert and Martin 1976; Lord et al. 1977). Results of drug discrimination studies have been consistent with hypothesized multiple populations of opioid receptors and have provided new insights into the actions of

opioid agonists and antagonists at the neuronal level (Herling and Woods 1981; Holtzman 1982a; Holtzman 1983).

STIMULUS PROPERTIES OF OPIOIDS THAT INTERACT WITH THE MU RECEPTOR

The discriminative stimulus effects of several types of opioid drugs appear to be mediated in whole or in part by the mu receptor. Included here are the traditional morphinelike agonists, partial morphine agonists, mixed agonist-antagonists, and at least some opioid peptides.

Morphinelike Agonists

The discriminative stimulus properties of morphine and related drugs have been characterized the most extensively of any group of opioids. Like many of their other effects, the discriminative stimulus effects of these drugs are robust and reproducible under a variety of experimental conditions. With minor exceptions, relationships among drugs are consistent and independent of the particular opioid used for training, species of experimental subject, or parameters of the discrimination paradigm (Holtzman 1983; Holtzman 1985a). There is now overwhelming evidence that the discriminative stimulus effects of these drugs are a receptor-mediated phenomenon, mediated by the same type of receptor that subserves other well-documented effects of the drugs, such as analgesia, depression of respiration, and induction of physical dependence (Holtzman 1982a; Holtzman 1983):

- all drugs usually classified as morphinelike agonists on the basis of their overall pharmacologic profile produce similar discriminative stimulus effects;
- they do so with an order of potency similar to that obtained in other bioassays involving interactions with the mu receptor (figure 1);
- steric requirements for morphinelike discriminative stimulus effects are the same as those for other mu receptor mediated effects, with strong selectivity for the levorotatory optical isomer of enantiomeric pairs;
- discriminative stimulus effects are blocked completely and in a surmountable manner by low doses of antagonists, such as naloxone and naltrexone, consistent with a competitive interaction at the mu receptor. (In the rat, apparent pA_2 values for interactions between naltrexone and morphine ┬Holtzman 1983) and between naloxone and morphine (Shannon et al. 1984) are 7.69±0.07 and 7.85±0.36, respectively, which accord well with the value of 7.95±0.19 for naloxone-morphine interactions in the tail compression test for analgesia (Tallarida et al. 1979)).

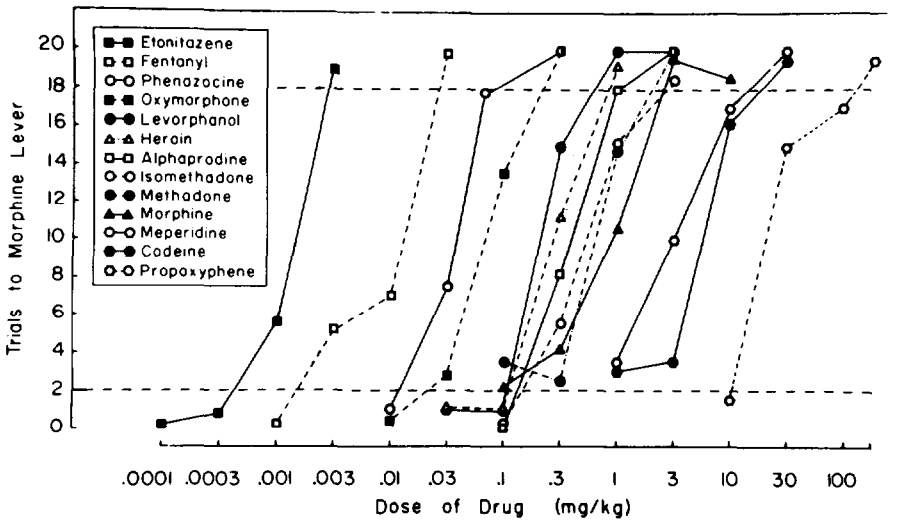

FIGURE 1. *Drugs classified as morphinelike agonists produced discriminative stimulus effects comparative to those of morphine, with the predicted order of potency*

NOTE: Drugs were administered SC 30 minutes before the start of a session to rats trained to discriminate between saline and 3.0 mg/kg of morphine In a two-choice discrete-trial avoidance paradigm. Each point is the mean number of trials completed on the morphine-appropriate lever in a 20-trial session; the remaining trials of the session were completed on the lever appropriate for the saline condition. Four or more rats were tested at each dose. The upper and lower broken horizontal lines indicate the minimum levels at which the discrimination performance of the animals was maintained during training sessions with 3.0 mg/kg of morphine and saline, respectively. Data are adapted from Shannon and Holtzman (1976; 1977b) and reproduced from Holtzman (1983). Copyright 1963, Academic Press, London.

Morphinelike discriminative stimulus effects are produced at central sites of action. The potency of morphine as a discriminative stimulus is fully two orders of magnitude greater when the drug is administered intracerebroventricularly (ICV) than when it is administered by systemic routes (Shannon and Holtzman 1977a). Furthermore, naltrexone methobromide, a quaternized analog of naltrexone that does not readily penetrate the blood-brain barrier, blocks the discriminative stimulus effects of morphine when it is administered ICV but not when it is administered systemically (Valentino et al. 1981; Locke and Holtzman 1985).

Partial Morphine Agonists

The few compounds available that are classified as partial morphine agonists have discriminative stimulus effects that. for all intents and purposes, are indistinguishable from those of the full agonists considered in the preceding section. Buprenorphine

probably has been the most widely studied drug of this class. Ranging from 10 to 100 times more potent than morphine, depending upon species, buprenorphine is reliably generalized to morphine in the rat (Shannon et al. 1984) and pigeon (France et al. 1984) and to etorphine in the rhesus monkey (Young et al. 1984). It occasions responding appropriate for the vehicle condition in animals trained to discriminate kappa or PCP/sigma agonists. The apparent pA_2 for the interaction between naloxone and buprenorphine in morphine-trained rats is 7.48±0.16, similar to values obtained for antagonist interactions with morphine. Thus, the discriminative stimulus effects of buprenorphine, like most of the other actions of the drug, are essentially exclusively morphinelike.

Mixed Agonist-Antagonists

In contrast to the full and partial morphine agonists, mixed agonist-antagonists have been viewed as having affinity for, but no intrinsic activity at, the mu receptor. These drugs have both affinity for and intrinsic activity at other receptor types. As a consequence, they should function as antagonists of drug effects mediated at the mu receptor while manifesting agonist activity that is mediated by one or more of the other postulated types of opioid receptors (Martin 1983). For example, pentazocine has been classified as a full agonist with low affinity for the kappa and PCP/sigma receptors, cyclazocine as a full agonist with high affinity for the kappa and PCP/sigma receptors, and nalorphine as a partial agonist at each of these two receptor populations (Martin et al. 1976). However, just as these drugs have many actions in common with morphine in human subjects (Martin 1967), they also share stimulus effects with morphine in animals. Morphine-trained rats generalize completely to pentazocine (Shannon and Holtzman 1976), and rats trained with pentazocine generalize completely to morphine (Kuhn et al. 1976). a symmetry in responding which provides compelling evidence of the commonality in the discriminative stimulus effects of the two drugs. In rats trained with morphine, the apparent pA_2 for the naltrexone-pentazocine interaction is 7.52±0.12 (Holtzman 1983). This finding suggests that the morphinelike discriminative stimulus effects of pentazocine are mediated by the same type of receptor with which morphine interacts: the mu opioid receptor.

Other mixed agonist-antagonists are generalized partially to morphine in the rat. That is, they occasion responding on the drug-appropriate lever that is significantly greater than that produced by vehicle but significantly less than that produced by the training drug. Included here are nalorphine, cyclazocine, and levallorphan (Shannon and Holtzman 1976; Shannon and Holtzman 1977b; Overton and Batta 1979), to name but a few. Systematic interaction experiments between morphine and nalorphine or cyclazocine yield good *in vivo* approximations of theoretical log-concentration-response curves for the combination of a partial agonist and a full agonist acting at the same receptor (figure 2). Thus, in the rat, many mixed agonist-antagonists appear, in fact, to be partial agonists at the mu receptor, not simply antagonists

at this site. Other lines of evidence derived from experiments in rats trained with fentanyl are consistent with the foregoing interpretation (Colpaert and Janssen 1984).

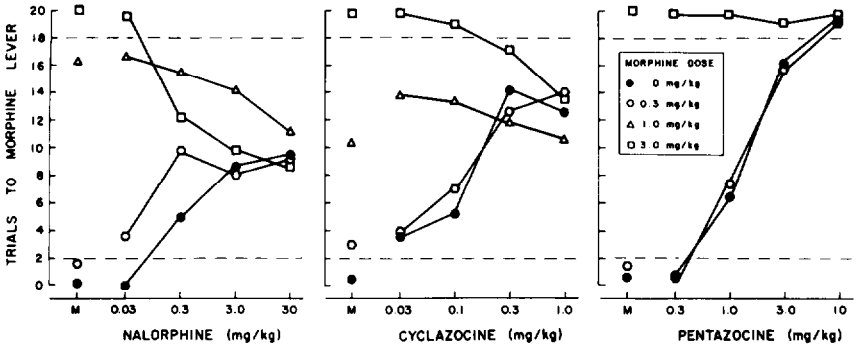

FIGURE 2. *Nalorphine and cyclazocine appear to be partial morphine agonists in the mt, whereas pentazocine acts as a full agonist*

NOTE: Stimulus generalization curves are shown for each agonist-antagonist tested alone (morphine dose: 0 mg/kg) or in combination with 0.3, 1.0, or 3.0 mg/kg of morphine In rats (n=5) trained to discriminate between saline and 3.0 mg/kg of morphine. The unconnected points above M indicate the mean number of trials completed on the morphine-appropriate choice lever In sessions that followed the administration of 0 (saline), 0.3, 1.0, or 3.0 mg/kg of morphine alone. Other details are the same as in figure 1. The family of curves for the combination of nalorphine and morphine and for cyclazocine and morphine converge upon the corresponding curve for nalorphine or cyclazocine alone, consistent with a partial agonist and a full agonist acting on the same receptor population (Arlens et al. 1964). Reproduced from Holtzman (1983). Copyrlght 1983, Academic Press, London.

In contrast to the robustness and uniformity of the discriminative stimulus effects of morphinelike agonists and partial agonists, stimulus effects of mixed agonist-antagonists are critically dependent upon factors such as the particular drug and dosage used for training and upon species of the experimental subject (Holtzman 1982a; Holtzman 1982b). Pentazocine, for example, is generalized only partially to morphine in the pigeon (Herling et al. 1980) and squirrel monkey (Schaefer and Holtzman 1981). Butorphanol and nalmexone, agonist-antagonists which have many pharmacologic characteristics in common with pentazocine in animals and humans (Griffiths and Balster 1979), exhibit similar species-dependent patterns of discriminative stimulus effects which are illustrated in figure 3. Such interspecies differences

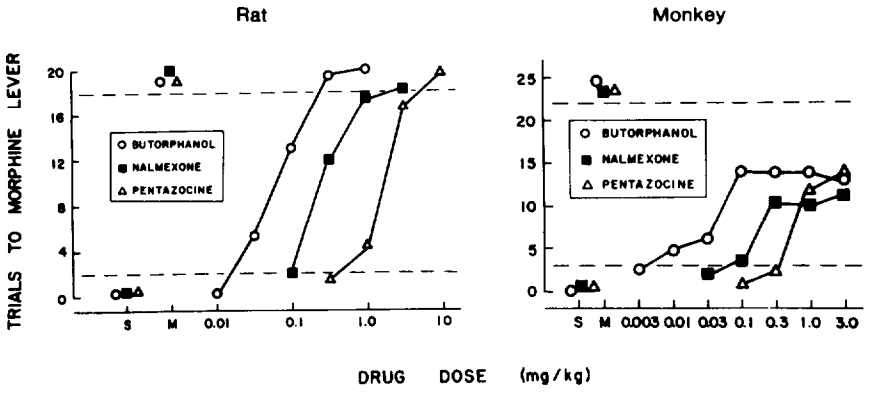

FIGURE 3. *The morphinelike discriminative stimulus effects of mixed agonist-antagonists are often species dependent*

NOTE: Butorphanol, nalmexone, and pentazocine were generalized to morphine completely by rats (n=4 or 5) but only partially by squirrel monkeys (n=3 or 4). Each species was trained to discriminate between saline (S) and 3.0 mg/kg of morphine (M) In a 20-trial (rats) or 25-trial (monkeys) session. Data are reproduced from Holtzmen (1985b). Copyright 1985, Elsevier Scientific Publishers, Ireland Ltd.

may well reflect differences in the densities and ratios of the several types of opioid receptors with which the agonist-antagonists interact. The rat brain is especially rich in mu receptors relative to other types of opioid receptors (Maurer 1982). The presence of "spare" mu receptors in the rat could permit some drugs that are partial agonists at the mu receptor, e.g., pentazocine, to appear as full agonists in this species.

Opioid Peptides

Data on discriminative stimulus effects of opioid peptides are sparse in comparison to the existing body of literature on the opioid alkaloids. This situation is due, in large part, to the technical difficulties inherent in maintaining healthy and behaviorally stable subjects during long-term experiments that require repeated injections of compounds into the brain. Some of these problems can be overcome by using opioid peptides that are metabolically stable so that they retain behavioral activity when administered systemically. To date, all such peptides have been found to have discriminative stimulus effects which are morphine-like, regardless of their relative selectivity for mu vs. delta receptors in binding assays or in bioassays on isolated tissue preparations. The metabolically stable analogs of methionine enkephalin, FK 33,824, which is selective for the mu receptor, and metkephamid, which shows selectivity for the delta receptor, were

generalized to morphinelike agonists when administered systemically to rhesus monkeys (Young et al. 1983). The putative delta receptor agonist D-ala , D-leu^5-enkephalin (DADL) was tested using the ICV route of administration in separate groups of rats trained to discriminate vehicle from either the mu receptor agonist fentanyl or the kappa receptor agonist ethylketocyclazocine. DADL was generalized to fentanyl at a dose of approximately 1.0 µg, but occasioned only vehicle-appropriate responding in the rats trained with ethylketocyclazocine (Shearman and Herz 1982a).

Locke and Holtzman (unpublished observations) examined a series of opioid peptides having relative selectivity either for the mu or delta opioid receptor. The peptides were tested ICV in rats trained to discriminate 3.0 mg/kg of morphine (SC) from saline. The mu-selective peptides (DAGO, FK 33,824, and morphiceptin), the delta-selective peptides (DADL and metkephamid), and ß-endorphin, which interacts equally well with both receptor types, all produced dose-dependent morphinelike stimulus control of behavior (table 1). These generalization data and the ease with which the morphinelike stimulus effects of the peptides could be blocked by naltrexone suggest that mu opioid receptors in the brain are critical for the genesis of morphinelike discriminative stimulus effects by these particular opioid peptides. It would be premature to dismiss entirely the possibility of a role for delta

TABLE 1. *Opioid peptides which produce morphinelike discriminative stimulus effects when administered intracerebroventricularty (ICV) to rate trained to discriminate between SC injections of 3.0 mg/kg of morphine and saline*[a]

Opioid	Relative Opioid Receptor Selectivity	Median Dose (ICV) Producing Morphine-like Stimulus Control of Behavior (nmol/rat)
D-Ala^2-NMePhe^4-Gly^5(ol) enkephalin (DAGO)	mu	0.02
D-Ala^2-NMePhe^{4}Met(O)^{5}ol enkephalin (FK 33,824)	mu	0.02
β-endorphin	mu and delta	0.3
D-Ala^2-NMeMet^5-NH_2 enkephalin (metkephamid)	delta	1.7
D-Ala^2-D-Leu^5 enkephalin (DADL)	delta	1.8
Morphine[b]	mu	10.5
Tyr-Pro-Phe-Pro-NH_2 (morphiceptin)	mu	58

[a]Three or more doses of each peptide were tested In each of four to seven rats (Locke and Holtzman, unpublished observations).

[b]Morphine was tasted over a range of doses in each of 27 rats (Locke and Holtzman 1985).

receptors in the discriminative stimulus effects of opioid drugs. Any such role, however, will remain obscure in the absence of studies on subjects trained to discriminate delta-selective agonists.

Clearly, studies of the discriminative stimulus effects of opioid peptides have only scratched the surface. Many peptides remain to be evaluated. The consistency of experimental outcomes across animal species must also be determined.

STIMULUS PROPERTIES OF OPIOIDS THAT INTERACT WITH THE KAPPA RECEPTOR

Opioids usually classified as kappa receptor agonists exhibit discriminative stimulus properties that are clearly different from those of drugs that interact primarily with the mu receptor. For example, rats trained to discriminate between saline and ethylketocyclazocine respond almost exclusively on the saline-appropriate lever when tested with graded doses of morphine or fentanyl; the same outcome is obtained when rats trained with fentanyl are tested with ethylketocyclazocine, bremazocine, or other prototypic kappa drugs (Shearman and Herz 1982b). The discriminative stimulus effects of kappa agonists are receptor mediated in the rat (Herling and Shannon 1982) and rhesus monkey (Hein et al. 1981; Young and Stephens 1984). satisfying the criteria of stereoselectivity, competitive blockade by antagonists, and order of potency among a series of related compounds (figure 4). The apparent pA_2 value for the interaction between naloxone and ethylketocyclazocine in ethylketocyclazocine-trained rats is 6.77 (Herling and Shannon 1982), significantly below the corresponding values for antagonist interactions with morphine (vide supra). Thus, the affinity of naloxone for the receptor mediating the stimulus effects of ethylketocyclazocine is approximately 10 times lower than its affinity for the receptor mediating the stimulus effects of morphine. This difference accords well with results from assays of radioligand binding to putative mu and kappa sites (Kosterlitz et al. 1981).

Full characterization of the stimulus control of behavior mediated by kappa receptors has been hampered by the lack of selective kappa antagonists as well as by the sometimes extreme differences in stimulus properties of drugs from species to species. Ethylketocyclazocine provides a good example of the problem. In the pigeon, the discriminative stimulus effects of this prototypic kappa agonist are entirely morphinelike (Herling et al. 1980; Herling and Woods 1981). The discriminative stimulus effects of nalorphine, too, vary markedly across species. Nalorphine is a partial mu agonist in the rat (figure 2) and also exhibits weak kappa activity in this species (Shearman and Herz 1982c), consistent with its earlier classification by Martin and colleagues (Martin et al. 1976; Gilbert and Martin 1976). In primates, however, nalorphine has no discriminative stimulus effects in common with morphine (Schaefer and Holtzman 1981). Indeed, it has a profile of stimulus properties entirely characteristic of a

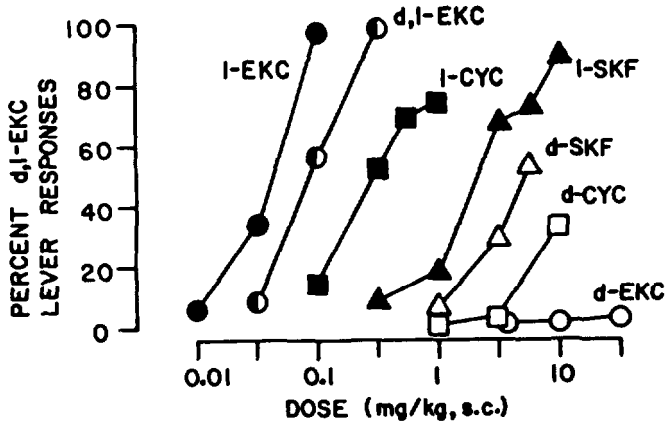

FIGURE 4. *Discriminative stimulus effects of the kappa receptor agonist ethytketocyclazocine (EKC) are stereo-selective for the levorotatory isomer and are mimicked by cyctazocine (CYC) and SKF-10,047 (SKF), with the predicted order of potency*

NOTE: Rats (n=5) were trained to discriminate between saline and 0.3 mg/kg of racemic EKC in a discrete-trial avoidance paradigm. Drugs were injected SC 15 minutes before the start of a 20-trial session. The ordinate is average percent of trials per session completed on the choice lever appropriate for EKC. Data are reproduced from Herling and Shannon (1982).

kappa receptor agonist. Nalorphine and ethylketocyclazocine generalize completely to one another in the rhesus monkey (Hein et al. 1981; Tang and Code 1983). The nalorphine cue is also generalized completely to other kappa agonists such as bremazocine, tifluadom, and U-50,488, with the predicted order of potency. Finally, stimulus control of behavior is selective for the levorotatory optical isomer of cyclazocine, SKF-10,047, and U-50,488, the member of the enantiomeric pair in which kappa activity resides (Tang and Code 1983).

Findings of the type described for nalorphine and the greater relative potency of kappa agonists in the squirrel and rhesus monkeys as compared to the rat--often two orders of magnitude--suggest that primates have a higher ratio of kappa to mu receptors than does the rat. Kappa sites represent only a small percentage of total opioid binding sites in rat brain (Maurer 1982). Perhaps this explains why dynorphin, postulated to be the endogenous ligand of the kappa receptor (Chavkin et al. 1982), and several of its biologically active fragments occasioned responding appropriate for the vehicle condition when tested ICV in rats trained

to discriminate between ethylketocyclazocine and saline (Shearman et al. 1985). It will be of interest to determine the discriminative stimulus properties of kappa-selective opioid peptides in a primate species.

STIMULUS PROPERTIES OF OPIOIDS THAT INTERACT WITH THE PCP/SIGHA RECEPTOR

A sigma receptor was proposed as one of the components of a three-receptor model of opiate activity derived from a series of experiments in the chronic spinal dog bioassay. Martin et al. (1976) suggested that the sigma receptor mediated the canine delirium induced by certain opioids such as SKF-10,047 (N-allylnormetazocine, NANM) and cyclazocine. The fact that both of these drugs are psychotomimetic in humans did not go unnoticed. Interest in the sigma site was stimulated further by the finding that saturable binding of the dissociative anesthetic phencyclidine (PCP) to brain preparations is displaced by nanomolar concentrations of NANM and cyclazocine (Zukin and Zukin 1979; Zukin and Zukin 1981; Quirion et al. 1981). Kappa receptor agonists are less potent in this regard; morphine and related mu agonists are essentially inactive, as are naloxone and naltrexone.

The discriminative stimulus effects of sigma opioids, like those of mu receptor agonists, are relatively robust, uniform across animal species, and independent of the drug used for training. The results of numerous studies are consistent with the notion that the stimulus effects of this group of drugs are mediated at a neuronal site that has not traditionally been associated with opiate activity, a site of action in common with PCP (table 2). Rats trained with either cyclazocine (Teal and Holtzman 1980) or NANM (Shannon 1983a) generalize completely to PCP and ketamine, another dissociative anesthetic and an analog of PCP. Conversely, rats (Holtzman 1980; Shannon 1981), squirrel monkeys (Holtzman 1982c), and pigeons (McMillan 1982) trained with PCP generalize completely to cyclazocine and NANM but emit responses appropriate for the vehicle condition when tested with a variety of mu or kappa agonists (figure 5). Stimulus control of behavior by these PCP/sigma agonists is essentially insensitive to blockade by even high doses of naloxone or naltrexone. In rats trained in a three-choice paradigm to discriminate concurrently between PCP, saline, and cyclazocine, the administration of a fixed dose of naltrexone in combination with high, graded doses of cyclazocine resulted in a dose-dependent increase in responses on the choice lever appropriate for PCP (White and Holtzman 1983). This outcome is compelling evidence that an opioid-antagonist-insensitive component of action of cyclazocine is responsible for the PCP/sigma-like discriminative stimulus effects of the drug.

In contrast to steric requirements for discriminative stimulus effects mediated by mu or kappa receptors (vide supra), the PCP/sigma site frequently favors the dextrorotatory conformation, although not consistently so. (+)-NANM is slightly more potent

TABLE 2. *Potency order of drugs for producing PCP- and d,l,-NANM (SKF-10,047)-like discriminative effects in the rat and for displacing binding of ^{3}H-PCP from rat brain membranes*

Drug	Training Drug:	Discriminative Effects		
		PCP[a] (2.0-3.0 mg/kg)	NANM[b] (3.0-5.0 mg/kg)	Binding[c]
l-Cyclaroclne		1	1	3
PCP		2	2	2
Dexoxadrol		3	3	1
d-NANM		4	4	4
d,l-Metazocine		5	8	—[d]
l-NANM		6	5	5
Dextrorphan		7	9	6
d-Cyclazocine		8	7	7
Ketamine		9	10	8
Levallorphan		10	6	—[d]

[a]From Holtzman (1980); Shannon (1982; 1983b).

[b]From Shearman and Herz (1982d); Shannon (1983a).

[c]From Mendelsohn et al. (1984).

[d]Not tested.

NOTE: Spearman rank correlation coefficient:

r_s =0.81 for column I vs. II ($p<0.005$)
r_s=0.90 for column I vs. III ($p<0.005$)
r_s=0.68 for column II vs. III ($p<0.005$)

than its levorotatory counterpart in producing PCP-like discriminative stimulus effects in a number of species (Brady et al. 1982a). On the other hand, (-)-cyclazocine is several times more potent than (+)-cyclazocine (Shannon 1982; McMillan 1982). The order of potency of the enantiomers of NANM and cyclazocine for producing PCP- and NANM-like discriminative effects in the rat parallels their order of potency in displacing H-PCP binding from membranes of rat brain cortex (table 2).

Some members of two other chemical families also have been found to produce PCP/sigma-like discriminative stimulus effects: opiate morphinans and nonopiate dioxolanes. Dextrorphan, the nonanalgesic enantiomer of the classical morphinelike agonist levorphanol has a profile of discriminative stimulus properties entirely characteristic of a PCP/sigma agonist (table 2 and Herling et al. 1983). Among the dioxolanes, etoxadrol, an intravenous anesthetic which produces psychotomimetic side effects in human subjects, and dexoxadrol, a structural analog, share discriminative stimulus effects with PCP, NANM, and dextrorphan in rats (Shannon 1982; Shannon 1983a), pigeons (Herling et al. 1983), and monkeys (Brady

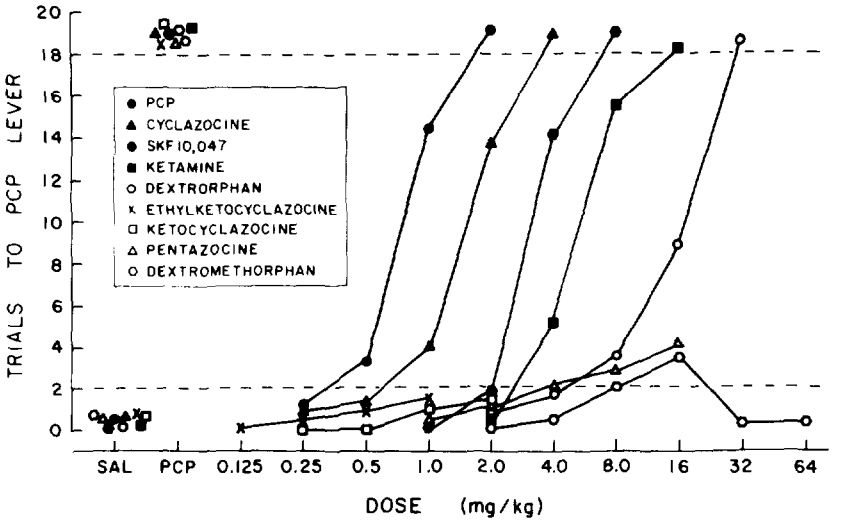

FIGURE 5. *Rats (n=5 or mope) trained to discriminate between IP injections of satins and 2.0 mg/kg of PCP generalize completely to ketamine and the opioids cyclazocine, SKF-10,047, and dextrorphan, but respond almost exclusively on the lever appropriate for the saline condition when tested with a variety of other opioids, some of which are illustrated here*

NOTE: Data are reproduced from Holtzman (1980). Copyright 1980, American Society for Pharmcology and Experimental Therapeutics.

et al. 1982b). Levoxadrol, the levorotatory isomer of dexoxadrol, is apparently devoid of PCP/sigma activity.

Cone et al. (1984) have proposed an elegant model of a receptor complex composed of several subsites with differing steric requirements. Separate subsites can bind PCP and its analogs, psychotomimetic opioids, and dioxolane derivatives. An important implication of this model is that the opioid structure is probably coincidental to the PCP-like stimulus properties of these drugs. Critical conformational features shared with PCP are most likely the principal determinants of the PCP/sigma activity of the opioids.

CONCLUSIONS

The discriminative stimulus properties of opioid drugs are diverse, consistent with concepts that multiple populations of receptors subserve the actions of these drugs. With the exception

of peptides, for which sufficient data are lacking, it is possible to partition opioids into three broad categories on the basis of their patterns of stimulus generalization to the various prototypic reference drugs used for training. Such categorization predicts sensitivity of discriminative stimulus effects to antagonism by naloxone or naltrexone and appears to reflect the sites of action of the drugs at the neuronal level (table 3). It should also be apparent from table 2 that the discriminative stimulus effects of opioids have significant parallels with other important actions of the drugs, for example, reinforcement efficacy in primate models of drug self-administration (Woolverton and Schuster 1983). Opioids with morphinelike discriminative stimulus effects are almost invariably reinforcing and are reliably self-administered. Opioids with discriminative stimulus effects in common with ethylketocyclazocine and nalorphine are rarely self-administered (Woods et al. 1982; Young et al. 1984). Opioids that engender PCP/sigma-like discriminative stimulus effects seem to fall between those two extremes (Slifer and Balster 1983).

TABLE 3. *Characteristics of opioids classified on the basis of their discriminative stimulus properties*[a]

	Complete Generalization to:		
	Morphine Fentanyl	Ethylketocyclazocine[b] Nalorphine[c]	SKF-10,047 (NANM) PCP
Proposed site of action (receptor)	mu	kappa	PCP/sigma
Sensitivity to naloxone or naltrexone	hlgh (<0.1 mg/kg)	moderete (>0.1 mg/kg)	low or none
Stereoselectivity	yes (1)	yes (1)	inconsistent or (d)
Consistency across animal species	high	low	moderate-high
Reinforcement efficacy (monkey)	high	low	moderate
Subjective effects (human)	“euphoria"	"dysphoria" (?)	psychotomimetic
Human abuse potential	high	none (?)	low-moderate (?)
Other drug examples	“classical” morphinelike opiates	ketocyclazocine bremazocine U-50,488	cyclazocine dextrorphan

[a]Modified from Holtzman (1985a; 1985b).

[b]In rats or primates.

[c]In primates.

Early suggestions of a correspondence between the discriminative stimulus effects of morphinelike agonists in animals and the

subjective effects of these drugs in humans also appear applicable to the discriminative stimulus and subjective effect of PCP/sigma agonists (Holtzman 1982d). The nature of the subjective effects produced by kappa agonists has not been fully determined. However, evidence is emerging that kappa receptors may subserve antagonist-sensitive dysphoric and perhaps even psychotomimetic symptomology in humans (Shannon 1982; Shannon 1983a; Tang and Code 1983). If the relationships between discriminative stimulus, reinforcing, and subjective effects suggested in table 3 are valid, the discriminative stimulus properties of opioids should afford important insights into components of drug action at the neuronal level that are directly relevant to abuse potential.

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Neurobiological Substrates of Drug Self-Administration

James E. Smith and Steven I. Dworkin

INTRODUCTION

Behavioral pharmacologists have been involved in investigations of the behavioral parameters influencing drug action for over three decades, with the basic assumption that these effects result from actions on brain neuronal systems. Today, it is generally accepted that the behavioral effects of many drugs result from alterations in neuronal activity through direct or indirect interaction of these substances with specific receptors. Neuroscientists are currently attempting to identify the loci of these actions and to characterize the neuronal systems that mediate these processes. This chapter will focus on the status of knowledge of the neuronal substrates involved in drug self-administration.

DRUG SELF-ADMINISTRATION: A COMPLEX BEHAVIORAL PHENOMENON

Drug self-administration is a complex behavioral phenomenon that likely encompasses many of the behavioral actions of the drug, including, but not limited to, the reinforcing effects. It is generally assumed that biological correlates of self-administration or experimental manipulations affecting drug intake primarily represent the reinforcing properties of the substance. However, the delivery of a drug, whether self-administered or not, results in a number of pharmacological actions. For example, opiates have multiple effects on the central nervous system that result in alterations in sensitivity to environmental stimuli, including decreased pain perception, footshock reactivity, separation distress and social bonding, and increased food and water consumption (Reid and Siviy 1983). Opiates also have stimulus- and/or response-modulating properties. The presence of the drug results in the initiation of neuronal events that are distinguished from those produced by drugs of different classes and sometimes even from others of the same class (Overton 1983). The presence of the drug may also initiate neuronal events that alter response rates, even drug-maintained responding (Goldberg and Kelleher 1977). Opiate self-administration most likely includes some of these CNS

effects. That the neuronal substrates reflect primarily the reinforcing properties is a limited assumption.

NEUROBIOLOGICAL MECHANISMS INVOLVED

Neurobiological substrates are defined here as the neuronal processes responsible for a specific action or function. The neurobiological substrates of opiate self-administration include all the neuronal events resulting in and from opiate self-administration. A complete understanding of these events requires knowledge of the neuronal circuits and pathways , activation or inhibition of which are necessary to these processes (figure 1). The location of the cell bodies and projections of each component neuron in such circuits is necessary, as is knowledge of the nature and localization of the necessary receptors. Knowledge of the influence of other inputs to each neuron participating in these circuits is also necessary for a complete understanding of the neuronal events mediating these processes. Needless to say,

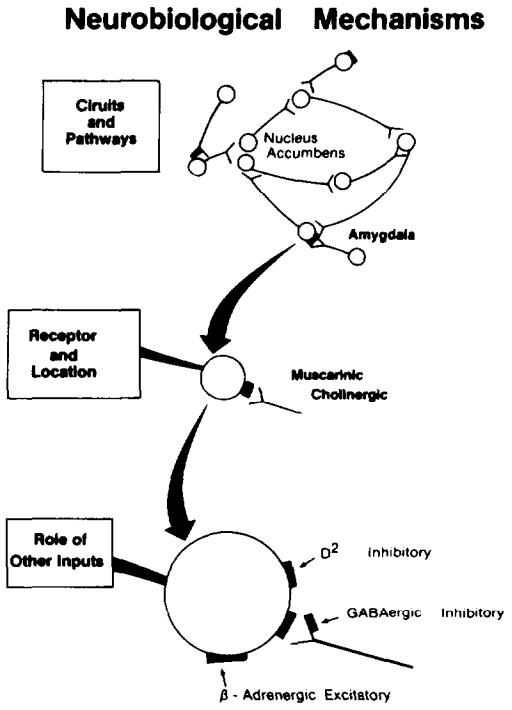

FIGURE 1. *Neurobiological substrates involve: (a) The identification of each neuron that participates in circuits mediating a behavioral effect; (b) identification of the nature and location of the receptors involved; (c) elucidation of the effects of other inputs to each participating neuron*

the neurobiological substrates of self-administration, or any other behaviors, are yet to be elucidated.

IDENTIFICATION OF NEUROBIOLOGICAL SUBSTRATES

As mentioned above, the neuronal systems affected by drug self-administration are likely to include many of the systems that control the behavioral actions of the drug. Neuronal circuits involved in the reinforcing effects, stimulus properties, response modulating effects, and other behavioral effects would most likely also be activated. This complicates the identification of neurobiological substrates exclusive to the reinforcing properties. Additional complications stem from the likelihood that there is some utilization of common neurons in the circuits that mediate any one behavioral effect (figure 2). For example, the circuits involved in the reinforcing properties of an opiate may include some neurons that also participate in circuits mediating the stimulus properties, response-rate altering, or consummatory behavior-modulating properties. Some participating neurons could also function in additional neuronal circuits involved in other behavioral effects. Moreover, unique subsets of neurons may mediate the same behavioral effect independent of the drug class. This behavior-specific function probably operates for reinforcing properties, since opiates and stimulants have both been shown to alter dopamine transmission. Thus, the neuronal circuits mediating the reinforcing effects of opiates may include some of the neurons that mediate the reinforcing properties of stimulants and/or benzodiazepines (figure 3). But some unique systems must also participate. Stereospecific binding sites for subclasses of

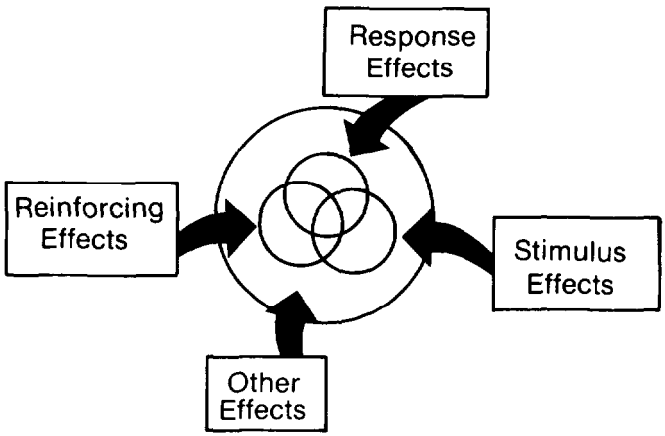

FIGURE 2. *The neuronal circuits involved in a particular behavioral drug effect may share some common neurons with circuits mediating other behavioral effects of the drug*

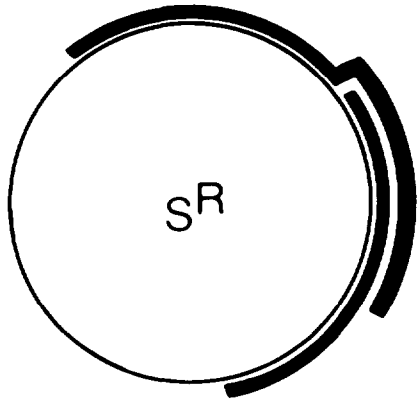

FIGURE 3. *The neuronal circuits involved in a behavioral effect of one drug class may share common neurons with circuits involved in the same behavioral effect of other drug classes*

opiates (Weber et al. 1982; Khachaturian et al. 1983), cocaine (Reith et al. 1980), amphetamine (Hauger et al. 1984), benzodiazepines (Braestraup and Squires 1977), and other CNS active drugs have been identified that are heterogenously distributed throughout the brain. If a common group of circuits mediates a specific behavioral drug effect, then the sites where a particular drug class initiates these activities may be separate and distinct (figure 4).

Identification of neuronal circuits involved in a specific behavioral drug effect is a difficult challenge. The degree to which a manipulation can similarly influence a behavioral effect exerted by several different drug classes points to commonalities in neurobiological mechanisms. Furthermore, these effects must be separated from manipulations that alter an animal's ability to respond (i.e., nonspecific motor effects). Appropriate control groups and experimental procedures are necessary to isolate and characterize the neuronal systems initiating such effects.

INTRAVENOUS SELF-AOMINISTRATION

Initial studies of the neuronal systems mediating intravenous drug self-administration utilized neurotransmitter receptor-blocking agents. The effects of such treatments on drug intake served as a rough estimate of the potential participation of that

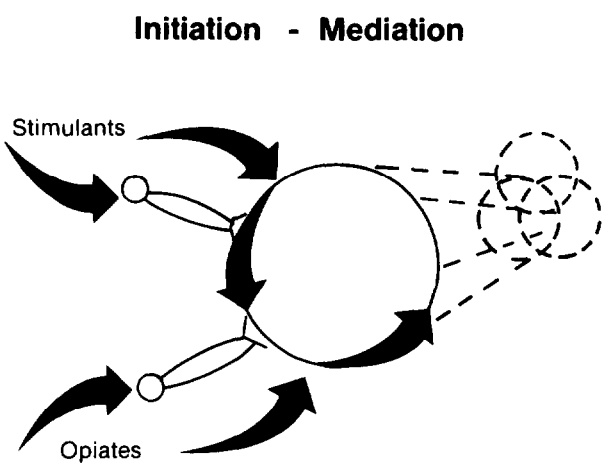

FIGURE 4. *Unique receptor-mediated sites for the initiation of a behavioral effect may exist for a drug that activates common circuits mediating the some behavioral effect for other drugs*

population of neurons in the processes responsible for self-administration. Pharmacological blockade experiments demonstrated cholinergic, dopaminergic, and noradrenergic neurons to be important to intravenous morphine-maintained responding, since atropine (Davis and Smith 1975; Glick and Cox 1975), haloperidol (Glick and Cox 1975; Smith and Davis 1973)) and dopamine-ß-hydroxylase inhibitors (Davis et al. 1975) all decreased intake. In contrast, haloperidol (DeWit and Wise 1977) and atropine (Wilson and Schuster 1973) decreased intravenous cocaine self-administration, while propranolol (Goldberg and Gonzalez 1976) increased it. Opiate-receptor antagonists also attenuated intravenous opiate self-administration, but did not affect cocaine intake, while doses of alpha-flupenthixol (a dopaminergic-receptor antagonist) that decreased cocaine intake did not affect heroin self-administration (Ettenberg et al. 1982). These kinds of experiments have generally suggested the involvement of cholinergic, dopaminergic, and noradrenergic neuronal systems in the neuronal processes maintaining intravenous opiate and stimulant self-administration. However, such systemic blockade experiments are extremely limited in localizing central mechanisms. A homogeneity of function of heterogeneous neuronal systems is assumed. Furthermore, the total effect is often concluded to be a specific result of alterations in reinforcing efficacy. These weak assumptions are intrinsic to this relatively nonspecific pharmacological manipulation.

Intracranial Lesions--Electrolytic

Lesion methodologies have also been used to identify brain regions or pathways that participate in a behavioral process. Electrolytic lesions of the brains of rats intravenously self-adminis-

tering morphine have been found to modulate drug intake. Lesions of the anterior cingulate cortex (Trafton and Marques 1971), frontal cortex, hippocampus, and medial raphe nucleus (Glick and Cox 1977) shifted the dose intake curve to the right, so that higher doses were necessary to maintain prelesion rates of intake; lesions of the caudate nucleus (Glick et al. 1975) and substantia nigra (Glick and Cox 1977) shifted the dose intake curve to the left, so that lower doses maintained prelesion self-administration rates. Lesions of the dorsal raphe nucleus, locus coeruleus (Glick and Cox 1977), amygdala (Glick and Ross 1983), medial thalamus, nucleus accumbens, posterior cortex, and olfactory tubercle (Glick and Cox 1978) had no effect on intake. Although electrolytic lesions are site-specific, they do not permit conclusions concerning the nature of the neuronal systems involved. Such lesions are also nonspecific and can destroy both excitatory and inhibitory systems in the same region, with no net change in drug intake occurring. This may result in the erroneous conclusion that a specific brain region is not involved in the processes responsible for drug-maintained responding.

Intracranial Lesions--Neurotoxins

Lesion techniques that involve the intracranial administration of neurotoxins into discrete brain regions can produce both site- and neuronal system-specific information. The intracranial administration of 6-hydroxydopamine (6-OHDA), after pretreatment with a noradrenergic reuptake inhibitor (i.e., desmethylimipramine), has been used to selectively destroy dopaminergic innervations of discrete brain regions. Such lesions of the nucleus accumbens decreased the intake of intravenous amphetamine (Lyness et al. 1979) and cocaine (Roberts et al. 1977; Roberts et al. 1980), but did not change heroin (Pettit et al. 1984), and produced only moderate increases in morphine intake (Smith et al., in press). Lesions (produced by 6-OHDA) of the ventral tegmental system, where the cell bodies of these mesolimbic-mesocortical dopaminergic neurons are localized, decreased intravenous cocaine intake (Roberts and Koob 1982).

These data collectively suggest a role for dopamine neurons in drug self-administration. However, not all mesolimbic or mesocortical dopamine projections are involved, since 6-OHDA lesions of the medial prefrontal cortex did not affect intravenous cocaine intake (Szostak et al. 1984). Other, nondopaminergic, neuronal systems may also be important. Kainic acid lesions of the nucleus accumbens, which primarily destroy interneurons and projections from the site of injection, also decreased intravenous cocaine self-administration (Zito et al. 1983), suggesting the involvement of elements postsynaptic to dopamine innervations. It was found that 5,7-dihydroxytryptamine lesions of serotonergic innervations of the nucleus accumbens did not affect intravenous amphetamine intake (Lyness et al. 1980), but had a profound effect on intravenous morphine self-administration (figure 5). Cholinergic innervations or projections from the nucleus accumbens may also be involved, since lesions with the neurotoxin AF64A shifted the

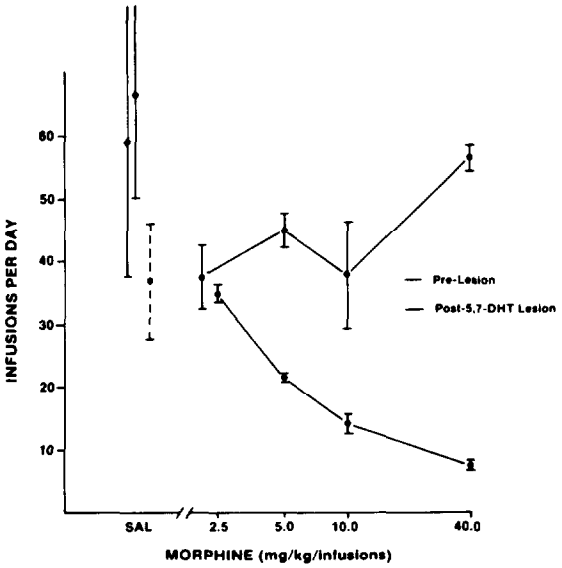

FIGURE 5. *The effects of bilateral 5,7-dihydroxytryptamine (5,7-DHT) lesions of the nucleus accumbens on intravenous morphine self-administration*

NOTE: The points are means and the error measures standard deviations for double determinations of 24-hour exposure probes of vehicle, 2.5, 5.0, or 40.0 mg/kg doses. Baseline drug exposure was 24-hour access to 10 mg/kg morphine. The 10 mg/kg points represent the mean of the baseline Intake on the days before dose probe manipulations. The lesion of serotonergic Innervations with 5,7-DHT (6 µg In 0.5 µl in each side after desmethyl-imipramine pretreatment) resulted in significant increases in morphine intake that was indistinguishable from vehicle intake. The point and dotted error line at saline represents intake with 7 continuous days of saline exposure postlesion and postdose intake analysis.

dose-intake curve for morphine to the right (figure 6). These data implicate multiple neuronal systems in the maintenance of drug self-administration and suggest some potential specificity of function with respect to drug class, since some lesions can affect the intake of one drug class but not that of another.

Intracranial Administration of Receptor Antagonists

Intracranial administration of receptor-blocking agents can potentially provide similar information on brain site and the nature of

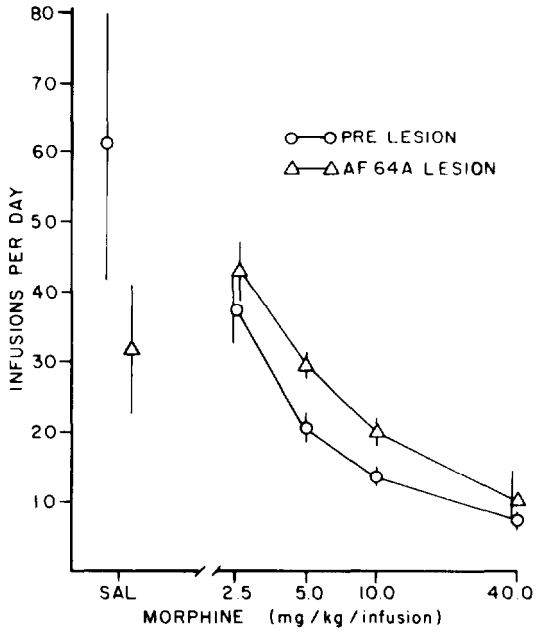

FIGURE 6. *The effects of bilateral AF64A lesions of the nucleus accumbens (2.3 μg each side in 0.5 μl) on intravenous morphine self-administration*

NOTE: Each point represents the mean and standard deviation of morphine intake for double determinations of 24-hour dose probes of vehicle, 2.5, 5.0, and 40.0 mg/kg/infusion, before and after such lesion In one animal. The lesion resulted in a shift to the right in the dose Intake curve, so that higher doses were necessary postlesion to maintain prelesion rates of self-administration.

participating neuronal systems. Infusion of the hydrophobic opiate-receptor antagonist, diallyl-nor-morphinium bromide, into the ventral tegmental area resulted in a dose-dependent increase in intravenous heroin self-administration, while similar infusions into the nucleus accumbens had no effect (Britt and Wise 1983). However, infusions of another slowly diffusing opiate receptor antagonist (methyl naloxonium chloride) into the nucleus accumbens resulted in dose-dependent increases in intravenous heroin intake, while infusions into the ventral tegmental area had no effect (Vaccarino et al. 1984). Although these data are contradictory, they do suggest a major role for opiate receptors in a particular brain area and the noninvolvement of receptors in another. The discrepancy in the findings from the two studies may be related to the different opiate receptor subtypes found in these two areas. This dissimilarity accounted for the disparity observed in opiate intracranial self-administration in these two brain regions (see below).

Neurochemical Measureaent

Neurochemical approaches have also been used to identify neuronal systems involved in drug self-administration. Concurrent measurement of the turnover rates of seven neurotransmitters (dopamine, norepinephrine, serotonin, aspartate, glutamate, glycine, and gamma-aminobutyric acid) in small brain regions of rats intravenously self-administering morphine, and in yoked morphine- and yoked vehicle-infused littermates has suggested the involvement of two neuronal circuits in the processes maintaining this behavior (Smith et al. 1982) (figure 7). Turnover rates of acetylcholine and muscarinic cholinergic binding were assessed in brain regions of separate groups of similarly treated littermates, demonstrating the involvement of specific cholinergic innervations (Smith et al. 1984a; Smith et al. 1984b). Integrating these neurochemical data, pharmacological blockade data, and data from lesion studies with current knowledge of neurotransmitter-specific pathways suggested two neuronal circuits to be involved in opiate self-administration. A frontal cortex/striatum/frontal cortex circuit (figure 8) and a nucleus accumbens/amygdala/entorhinal cortex/hippocampus/nucleus accumbens circuit (figure 9) were identified. Cholinergic innervations of the frontal cortex from the globus pallidus, from

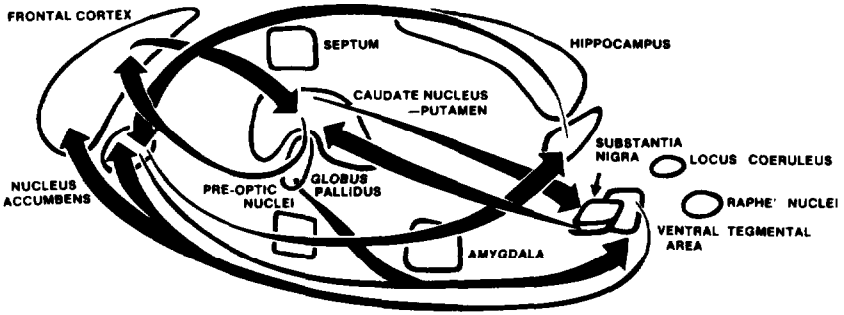

FIGURE 7. *Circuits proposed to mediate intravenous morphine self-administration*

NOTE: The circuits include a hippocampal formation/nucleus accumbens/amygdalold complex/entorhinal cortex/hippocampal formation circuit and a frontal cortex/striatum/frontal cortex circuit. Activity in these circuits may be modulated by the substantia nigra, ventral tegmental area, locus coeruleus, and raphe nuclei. Feedback pathways to these brainstem nuclei may In turn modulate activity in these centers.

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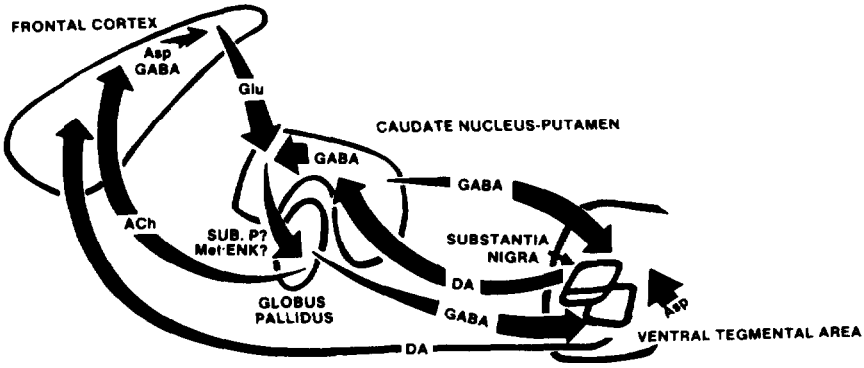

FIGURE 8. *Frontal cortex circuit proposed to partially mediate opiate reinforcement*

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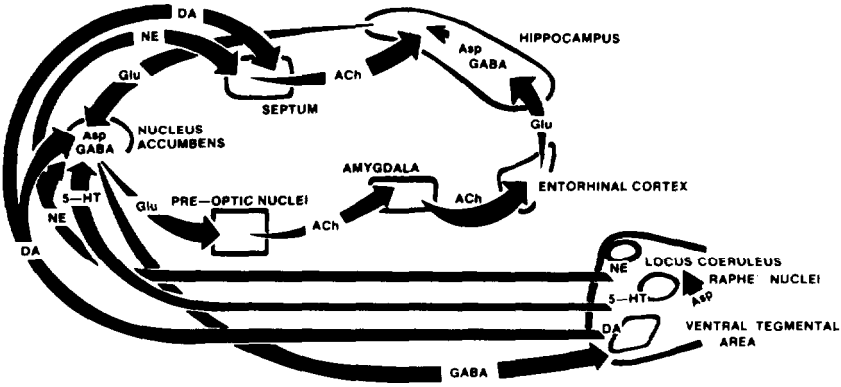

FIGURE 9. *Hippocampal circuit proposed to partially mediate opiate reinforcement*

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the preoptic region to the amygdala, from the amygdala to the entorhinal cortex, and from the septum to the hippocampus were implicated, as well as glutamatergic innervations of the striatum from the frontal cortex, to the nucleus accumbens from the hippocampus, and to the preoptic region from the nucleus accumbens. Aspartergic interneurons or innervations of the hippocampus, nucleus accumbens, and brainstem also appear to be involved, as do intrinsic GABA neurons in the frontal cortex, hippocampus, nucleus accumbens, and striatum. Apparently, GABA feedback pathways to the brainstem from the striatum and nucleus accumbens are also involved. Dopamine innervations of the striatum, frontal cortex, nucleus accumbens, and septum also appear to participate, as do serotonergic innervations of the nucleus accumbens and noradrenergic innervations of the nucleus accumbens and septum.

These circuits may be generalized reinforcement pathways, since autoradiographic analysis of the accumulation of radioactive 2-deoxyglucose in animals intracranially electrically self-stimulating one of two different brain regions (ventral tegmental area or substantia nigra) have identified the involvement of many of the same areas as these neurotransmitter turnover rate studies (Esposito et al. 1984; Porrino et al. 1984). If such general reinforcement systems exist, then areas and receptors that initiate activity in these systems may also exist. Intracranial self-administration methodologies can be used to identify and characterize such initiation systems.

INTRACRANIAL SELF-ADMINISTRATION

If a substance is delivered into a brain region where its receptors are localized and this delivery results in the initiation of reinforcing neuronal activity, this substance would be expected to be self-administered into this region. However, the injection of a substance directly into the brain could modify neuronal activity nonspecifically. Chemicals can initiate neuronal activity in a nonselective manner similar to that produced by electrical stimulation. Thus, self-administration of a drug could actually be chemically elicited stimulation of little behavioral relevance. Such misinterpretations can be avoided by using physiological concentrations and demonstrating that the self-administration is receptor mediated. Selective receptor antagonists should be used to attenuate intake as has been done with intravenous self-administration (Woolverton and Goldberg 1984). Furthermore, an intracranially infused substance could elicit a motor reflex nonspecifically or increase motor activity (Costall et al. 1982); either could result in elicitation of additional responses that result in more infusions, thus continuing the process. These drug-elicited responses are not emitted operant responses whose frequency has increased because of reinforcing consequences. Such behavior, therefore, should not be interpreted as self-administration. Schedules of drug presentation can be used to prevent such elicited behavior from resulting in repeated drug infusion.

Some brain regions support intracranial self-administration of opioids and stimulants while others do not. Morphine is self-administered into the ventral tegmental area at 150 pmole doses, but is not self-administered into the nucleus accumbens, caudate nucleus-putamen, periventricular gray substance, or lateral hypothalamus (Bozarth and Wise 1981). These data resulted in the hypothesis that the site of opiate reinforcement after systemic injection is the cell bodies of the mesolimbic mesocortical dopamine system in the ventral tegmental area. But, self-administration of similar doses also occurs in the septum, preoptic region, anterior hypothalamus, and lateral hypothalamus (Stein and Olds 1977), and much information exists concerning the heterogeneous distribution of opiate receptors and the relative affinity of various agonists for them. The nucleus accumbens contains primarily delta opiate receptors, and the ventral tegmental area, primarily mu opiate receptors (Goodman et al. 1980). It is not surprising that a dose of a mu agonist (morphine) is self-administered into a brain region where these receptors predominate and not into another brain region where delta receptors predominate. Morphine has a lower affinity for the delta receptor. Accordingly, higher doses are necessary to produce an equivalent degree of delta receptor binding. Higher doses of morphine have been found to be self-administered into the nucleus accumbens (Olds 1982), as are higher doses of the delta opiate agonist, met-enkephalin (Goeders et al. 1984). Thus, opioids will initiate reinforcing neuronal activity in brain regions other than the ventral tegmental area. Intracranial self-administration should be expected to be dose related, as is intravenous and oral self-administration. If dose-intake curves were routinely generated, then such erroneous conclusions could often be avoided. Self-administration of methionine enkephalin into the nucleus accumbens is dose related, with maximal intake at 500 pmole (figure 10). This self-administration can also be placed on intermittent schedules, with the animals increasing response output to maintain consistent infusion intervals (figure 11). The self-administration is probably emitted reinforced behavior, not elicited responding, since the animals will respond on an active lever but not on an inactive lever, and will change their responding appropriately when the contingency is switched between the levers (figures 11 and 12). This self-administration is also receptor mediated, since naloxone will attenuate intake to vehicle levels (figure 13). In summary, opioids appear to initiate neuronal activity in specific brain regions where appropriate opioid receptors are localized and such neuronal activation involves general reinforcement circuits.

Stimulants are also intracranially self-administered into discrete brain regions. Amphetamine is self-administered into the nucleus accumbens at 5 nmole doses (Hoebel et al. 1983) and cocaine into the medial prefrontal cortex at 50 to 100 pmole doses (Goeders and Smith 1983). Neither the nucleus accumbens nor the ventral tegmental area support cocaine self-administration at a range of doses (25 pmole to 5 nmole) (Goeders and Smith 1983),

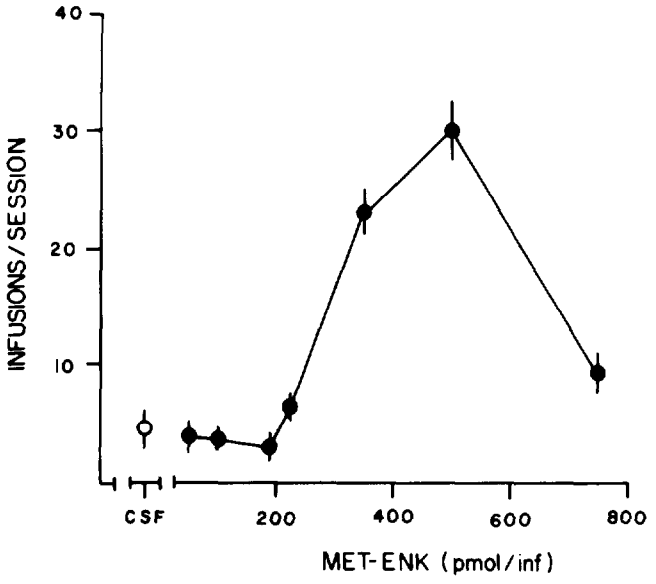

FIGURE 10. *Dose intake relationship for the intracranial self-administration of vehicle and methionine enkephalin into the nucleus accumbens on a fixed ratio 1 schedule of reinforcement*

NOTE: Points are means and the error measures standard deviations for double determinations In seven rats.

SOURCE: Goeders et al. 1984, Copyright 1984, Ankho International, Inc.

supporting the existence of specific sites in the brain for initiation of reinforcing neuronal activity. The self-administration of cocaine into the medial prefrontal cortex appears to be mediated by a direct action of cocaine on presynaptic dopamine nerve endings, since 6-OHDA lesions of this region will attenuate drug intake (Goeders and Smith, in press). Postsynaptic D_2 dopaminergic receptors are also involved since sulpiride will attenuate intake in intact animals (Goeders and Smith 1983), and dopamine, when substituted for cocaine, will initiate self-administration after 6-OHDA lesions (Goeders and Smith, in press).

If neuronal circuits mediate reinforcement processes, then it should be possible to initiate this activity with exogenous administration of endogenous substances that may be released by neurons participating in these processes. Rats have been demonstrated to self-administer neurohumors directly into brain regions where they

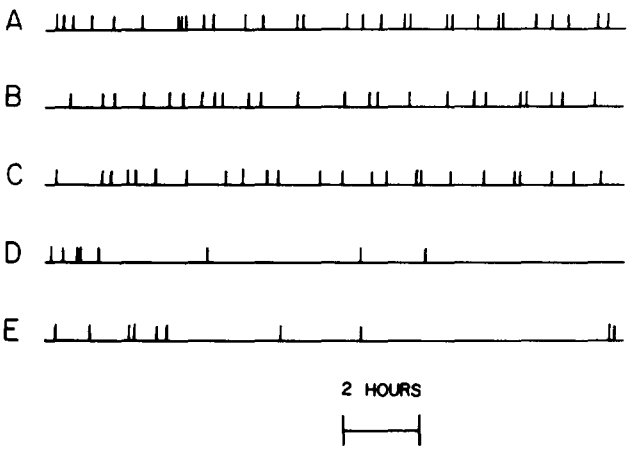

FIGURE 11. *Patterns of self-administration of 500 pmole doses of methionine enkephalin into the nucleus accumbens for one animal exposed to* four *experimental conditions*

NOTE: The conditions were (A) a fixed ratio 1 schedule; (B) a fixed ratio 5 schedule; (C) and (D) active and inactive lever presses during a two-lever discrimination experiment; (C) infusions from responding on the active lever on a fixed ratio 1 schedule; (D) nonreinforced responses on the inactive lever; and (E) infusions of vehicle (artificial CSF) on a fixed ratio 1 schedule.

SOURCE: Goeders et a I. 1984, Copyright 1984, Ankho International, Inc.

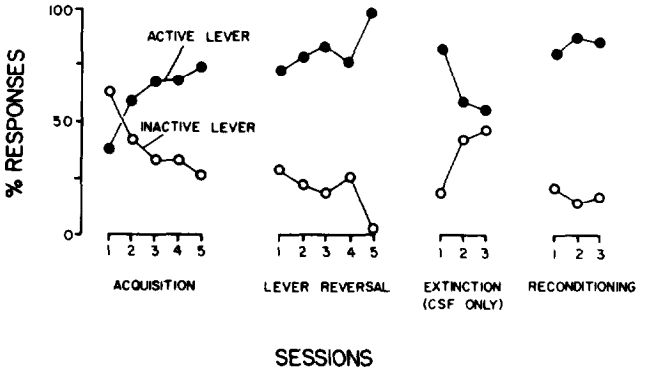

FIGURE 12. *Percent responding maintained by intracranial infusions of 500 pmole of methionine enkephalin into the nucleus accwnbens in a two-lever choice procedure on a fixed ratio 1 schedule*

SOURCE: Goeders et al. 1984, Copyright 1984, Ankho International, Inc.

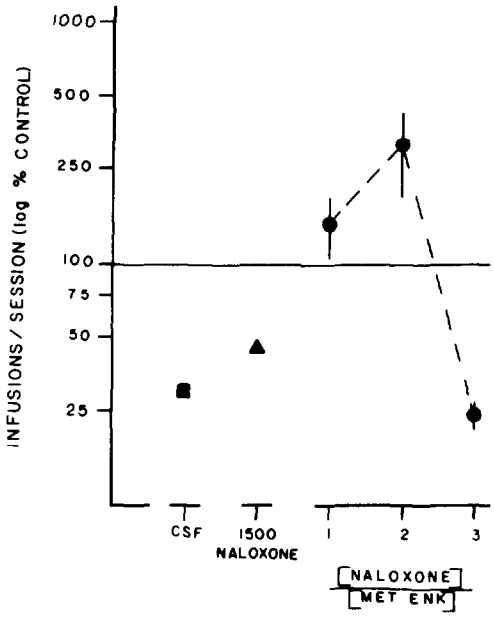

FIGURE 13. *Effects of naloxone on methionine enkephalin self-administration into the nucleus accumbens presented as the percent responding maintained by 500 pmole of methionine enkephalin (100 percent)*

NOTE: Including equimolar or twice equimolar concentrations of naloxone resulted in a transient increase in intake, while tripling the molar ratio of naloxone to methionine enkephalin resulted In extinction. Points are means and error measures standard deviations for double determinations in two animals.

SOURCE: Goeders et al. 1984, Copyright 1984, Ankho InternatIonal, Inc.

are released endogenously. Methionine enkephalin is self-administered into the nucleus accumbens (Goeders et al. 1984) as are dopamine (Dworkin et al., in press) and cholecystokinin (Hoebel and Aulisi 1984), while neurotensin is self-administered into the ventral tegmental area (Glimcher et al. 1983). These substances are released by neurons in these regions, suggesting that endogenous release may occur when reinforcement circuits are activated.

CONCLUSIONS

Specific neuronal pathways and circuits likely mediate reinforcement processes, as demonstrated by lesion studies and neurotransmitter turnover rate and receptor binding studies in intravenous drug self-administering rats. Multiple neurohumors are likely involved in these circuits and in the mediation of activity therein. It is likely that specific receptors in discrete brain regions are

the sites of initiation of reinforcing neuronal activity, as demonstrated by the intracranial self-administration studies. Cocaine will initiate such reinforcing neuronal activity at specific brain regions but not at others. Activation of these initiation sites likely results in neuronal activity in reinforcement circuits that may be involved in general reinforcement processes, as demonstrated by the autoradiographic distribution of 2-deoxyglucose in animals intracranially self-stimulating. Investigations in progress to identify neurohumors and brain regions that initiate reinforcing neuronal activity should lead to a better understanding of the neurobiological substrates of drug self-administration. Furthermore, similar techniques can be employed to investigate the neurobiological mechanisms involved in other behavioral drug effects.

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Neurochemical Substrates for Opiate Reinforcement

George F. Koob, Franco J. Vaccarino, Marianne Amalric, and Floyd E. Bloom

INTRODUCTION

Drugs such as the psychomotor stimulants and opiates act like other natural reinforcers that increase the probability of a response: they strengthen and maintain operant behavior, e.g., drug self-administration (Pickens and Harris 1968; Woods and Schuster 1968; Deneau et al. 1969; Thompson and Pickens 1970; Yokel and Pickens 1973; Schuster and Thompson 1969). The repeated use of opiate drugs in man is thought to be based both on the pleasurable effects of the drugs and on their effects in terminating the pattern of opiate withdrawal. Although there is some argument over the relative importance of these two factors, tolerance and physical dependence have long been held to be major motivators for continued administration of opiates (Lindesmith 1968). Others have argued that an essential component of opiate abuse is the direct reinforcing effect of the drug, or its "euphorigenic" effect (Kornetsky and Wheeling 1982). Indeed, humans will repetitively use opiates in the absence of physical dependence (Zinberg et al. 1978; Harding and Zinberg 1983). Rats will also readily self-administer heroin without prior physical dependence and can maintain indefinitely this self-administration with limited daily access to heroin, without the development of obvious physical dependence (Koob et al. 1984). This "euphorigenic" effect of opiates has been linked to a direct action on brain reinforcement systems (Marcus and Kornetsky 1974; Kornetsky et al. 1979; Wise 1980). Indeed, the neuropharmacological advances that allow for relatively specific inactivation of specific neurochemical systems make it feasible to examine the neurobiological mechanisms for these reinforcing effects. Such studies not only provide information on the basic mechanisms of action for the behavioral effects of these drugs, but also may provide insight into the neurobiological organization of reinforcement processes themselves.

In stimulant and opiate self-administration, animals maintain a relatively stable level of drug intake over time, with very regular interinjection intervals, particularly with short daily sessions (approximately 3 hours) (Ettenberg et al. 1982; Koob et

al. 1984). In response to changes in injection dose, animals typically show an inverse relationship between dose and number of injections per session; i.e., low doses produce a higher number of self-injections than do higher doses (figure 1). More important for the present context is the nature of the change in behavior following treatment with a pharmacological antagonist. Treatment with the opiate receptor antagonist produces an increase in the number of self-injections of morphine (Goldberg et al. 1971; Weeks and Collins 1976; Ettenberg et al. 1982). This increase is generally considered to reflect a competitive functional interaction; the rat presumably increases its drug self-administration to compensate for the decreased effectiveness, in the presence of partial receptor occupancy by the antagonist, of the opiate as a reinforcer. Consequently, an increase in self-administration resulting from administration of an opiate antagonist is qualitatively similar to the effects of decreasing the dose of drug per injection. A hypothetical description of this relationship is shown in figure 2.

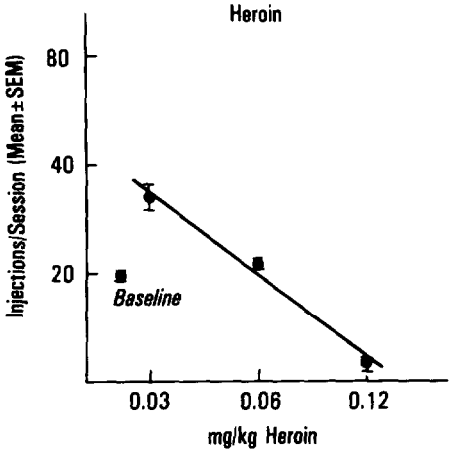

FIGURE 1. *Dose-response relationship for heroin self-administration in rats*

NOTE: Rats were allowed dally 3-hour access to heroin (0.06 mg/kg/injection) on a continuous reinforcement schedule. After stable baseline responding (± 20 percent mean for 3 days) was established, rats were subjected to a doubling of the dose. followed by a return to baseline. followed by a halving of the dose on 3 successive test days. Results are expressed as mean ± S.E.H., n=4.

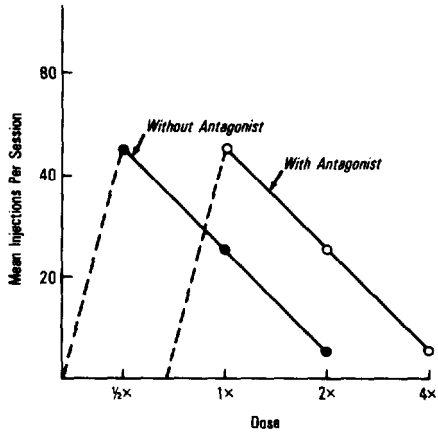

FIGURE 2. *Hypothetical model of the effects of a pharmacological antagonist on the dose-response function with self-administration*

NOTE: Note the shift of the dose-response function to the right. With this model, a decrease in reinforcement value Is reflected in an increase in the number of self-administered drug injections.

NEUROCHEMICAL SUBSTRATES OF HEROIN REINFORCEMENT

The Role of Peripheral Opiate Receptors

In humans, opiate injection is accompanied by "a warm flushing of the skin and sensations in the lower abdomen described by addicts as similar in intensity and quality to sexual orgasm" (Jaffe 1980; Wikler 1952). Known as the "rush," this sensation lasts for approximately 45 seconds and is generally thought to be one of the motivating factors involved in opiate use (Jaffe 1980). However, it is not clear that this rush is mediated by direct drug action on the central nervous system (CNS), since it is well established that interoceptive autonomic stimuli have an important role in the maintenance of heroin consumption in humans (Meyer and Mirin 1979). More operationally, it is not known whether the reinforcing properties of opiates that result from the sensations associated with this rush arise directly from activation of opiate receptors in the CNS or from opiate receptors localized in the periphery.

In light of the above, we compared the potency and efficacy of systemic administration of naloxone and naltrexone with their quaternary derivatives in antagonizing the reinforcing properties

of heroin. The quaternary derivatives of naloxone and naltrexone were chosen because of their potentially selective antagonist action, which excludes them from penetrating through the blood brain barrier. As a result, they can antagonize opiate effects on peripheral opiate receptors, as inferred from their ability to antagonize morphine effects on gastrointestinal transit, but do not antagonize central opiate actions on pain (Tavani et al. 1979). Recent studies have confirmed, conditionally, the peripheral selectivity of these compounds (Bianchi et al. 1982). Furthermore, others have reported that quaternary naltrexone was ineffective as an antagonist of the morphine discriminative stimulus or in precipitating abstinence in morphine-dependent rhesus monkeys (Valentino et al. 1981).

In rats self-administering heroin (0.06 mg/kg/injection in daily 3-hour sessions), low doses (0.05 to 0.2 mg/kg) of naloxone and naltrexone produced dose-dependent increases in self-administration; at higher doses (10 to 30 mg/kg), these drugs produced transient decreases in heroin self-administration, followed by recovery (Ettenberg et al. 1982; Koob et al. 1984) (figure 3).

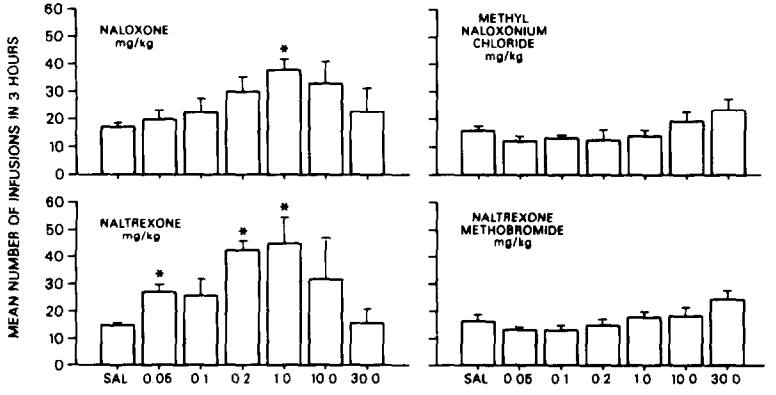

FIGURE 3. *Effects of naloxone, methylnal oxonium chloride, naltrexone, and naltrexone methobromide on responding over the total 3-hour test session for rats self-administering heroin*

NOTE: Data are expressed as mean ± S.E.M. of the total Infusions for 3 hours. Asterisks Indicate that the-treatment doses were reliably different from the saline (SAL)-pretreatment condition, $p<.05$ (paired t-test). Note that the SAL group represents mean t S.E.M. of al I the SAL observations (which Is equal to the number of different rats used in each drug group) for that drua: i.e., for naloxone. n=14: for methylnaloxonium chloride. n=15: for naltrexone; n=9; and for naltrexone methobromide, n=11. Each dose consists of five separate observations. Koob et al. 1984. Copyright 1984, Williams & Wilkins Company.

The quaternary derivatives were ineffective as antagonists of heroin self-administration in doses 200 times greater than the effective antagonist dose of naloxone or naltrexone (Koob et al. 1984). These results support the hypothesis that the acute reinforcing properties of intravenous (IV) opiates associated with the sensation of the rush involve opiate receptors located within the central nervous system (CNS) and do not involve peripheral opiate receptors (Koob et al. 1984).

In support of a selective peripheral action for these quaternary compounds, others have shown that the quaternary derivatives of opiate antagonists do not induce withdrawal signs in morphine-dependent dogs or monkeys at doses that blocked morphine-induced intestinal spike potentials (Russell et al. 1982). Also, the present results are unlikely to be explained simply on the basis of differential binding potency of the antagonists to opiate receptors. For example, methylnaloxonium (MN) appears to be one-tenth as potent as naloxone in displacing tritiated etorphine binding in rat brain homogenates (de Graaf et al., unpublished), and data from our laboratory indicate that MN is one-eighth as potent as morphine in displacing tritiated DAGO binding in rat brain homogenates and one-eleventh as potent as naloxone in displacing tritiated DAGO binding (Koob et al., unpublished). These results suggest that MN does block opiate receptors in a manner similar to that of naloxone itself. In addition, others have shown in vivo that the dose (1 mg/kg) of naloxone methobromide required for complete antagonism of the effect of morphine (5 mg/kg) on gastrointestinal transport in rats was only two to four times higher than the dose of naloxone hydrochloride required to produce the same effect (Bianchi et al. 1982). Although not directly evaluated in our experiments, these potency ratios suggest that the quaternary derivatives are effective opiate antagonists in the periphery.

The Role of Brain Dopamine Receptors

Previous studies have shown that centrally acting opiate receptor antagonists increase heroin self-administration, and centrally acting dopamine receptor antagonists increase cocaine self-administration. Unknown was whether or not these effects were pharmacologically independent of each other. To examine whether dopamine receptor antagonists alter the self-administration of opiates, rats trained to self-administer heroin (0.06 mg/kg/injection) intravenously for daily 3-hour sessions were subjected to a series of doses of the dopamine receptor antagonist alpha flupenthixol. Alpha flupenthixol, in doses that dose-dependently increased cocaine self-administration, failed to increase heroin self-administration. Indeed, the only significant effect on heroin self-administration was a decrease in self-administration at 0.4 mg/kg of alpha flupenthixol, a cataleptic dose. Naltrexone produced a dose-dependent increase in self-administration but had no effect on cocaine self-administration (Ettenberg et al. 1982). These results confirmed those observed by others using other

antagonists of opiates and dopamine (Goldberg et al. 1971; Yokel and Wise 1976; Weeks and Collins 1976; De Wit and Wise 1977).

The increased responding observed with the respective antagonist for each drug is thought to occur because the antagonist drugs presumably compete with the same synaptic sites as those influenced by the self-administered drug. Heroin, for example, is assumed to be converted to morphine in the brain, and the morphine is assumed to bind to central opiate receptors (Way and Adler 1960). Cocaine is thought to enhance dopaminergic transmission by blocking the reuptake of presynaptic dopamine (Patrick et al. 1975). Therefore, the net reinforcement produced by combining the self-administered drug and its specific antagonist is equivalent to the net effect on neurotransmission; animals respond to increasing doses of the antagonist by increasing the amount of drug self-administered. Of particular significance was the observation that low doses of alpha flupenthixol did not increase responding for heroin, nor did naltrexone increase cocaine-reinforced responding. The specificity with which these antagonists exerted their behavioral effects strongly suggests that separate neural substrates are responsible for the reinforcing actions of heroin and cocaine (Ettenberg et al. 1982).

Location Within the CNS of the Opiate Receptors Critical for Heroin Reinforcement

The mesolimbic dopamine system has not only been accorded an important role in psychomotor stimulant reward; it has also been hypothesized that this system is critical for the reinforcing properties of opiates (Bozarth and Wise 1981a). For example, rats will self-administer morphine into the ventral tegmental area (VTA) (Bozarth and Wise 1981b), and more recent studies have shown that rats will directly self-administer D-ala^2-methionine enkephalin into the nucleus accumbens (Goeders et al. 1984). Indeed, Britt and Wise (1983) have shown that administration of quaternary nalorphine (diallyl nor-morphinium bromide) into the VTA but not into the nucleus accumbens (N.Acc.) attenuated the self-administration of heroin. Our next series of studies was designed to extend these observations by examining the effects of methylnaloxonium chloride, a naloxone derivative known not to cross the blood brain barrier on IV heroin self-administration after microinjection into the cerebral ventricles, the VTA, or the N.Acc.

Rats were implanted with intracerebral injection cannulas aimed above the lateral ventricle, the VTA, or the N.Acc. They were trained to self-administer heroin (0.06 mg/kg/injection) intravenously. Following stable responding, each rat received bilateral microinjection of methylnaloxonium chloride (MN) into the lateral ventricle, the N.Acc., or the VTA 10 minutes prior to self-administration tests.

The results showed that lateral ventricular injections of MN produced a dose-dependent increase in heroin self-administration similar to that observed for systemic injections of naloxone

(figure 4). Effective doses ranged from 1.0 to 4.0 µg (Vaccarino et al. 1985a). Similar results were obtained following injections of MN into the VTA with no effect until MN reached a dose of 1.0 µg (figure 5). However, MN injected into the N.Acc. was approximately eightfold as potent at increasing self-administration of heroin. Significant increases were observed in doses from as low as 0.125 to 0.25 µg, with peak effects at 0.5 µg (figure 6) (Vaccarino et al. 1985b). MN had no effect on cocaine self-administration.

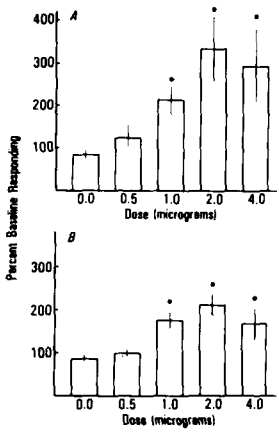

FIGURE 4. *The effects of intracerebroventricular (ICV) MN treatment on responding for heroin over the first hour (A) and over the total 3-hour self-administration session (B)*

NOTE: Response rates were expressed as the percentage of baseline responding. Asterisks indicate that the treatment doses were significantly different from the saline treatment, $p<.05$ (Newman-Keuls test). Six rats were tested across all drug treatments. The day prior to ICV Injections was used as the baseline day. Vaccarino et al. 1985a. Copyright 1985, Ankho International Inc.

The net results suggest that the N.Acc. is an important and possibly critical substrate for the reinforcing actions of opiates. Effective doses of MN in the N.Acc. were approximately one-eighth those observed for lateral ventricular injections. Injections of MN into the VTA were no more effective than lateral ventricular injections. These results suggest that the receptors important for opiate reward may be localized on neurons in the region of the N.Acc., as well as in the VTA, as hypothesized by Bozarth and Wise (1981a). To address the possibility that these receptors were localized on dopamine neurons terminating in the N.Acc., subsequent work in our laboratory examined whether destruction of presynaptic dopamine terminals in the N.Acc. would alter heroin self-administration.

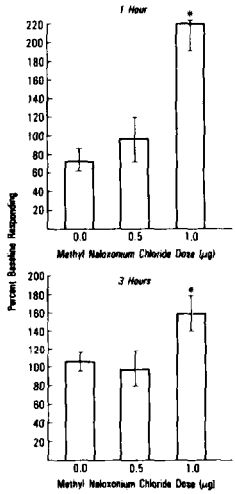

FIGURE 5. *Percent baseline (predrug day) responding for IV heroin during the first hour (top graph) and for the total 3 hours (bottom graph) of the heroin self-administration session following MN injections into the ventral tegmentat area*

NOTE: Asterisks indicate a significant difference ($p<.05$) from both saline vehicle (0.0 dose) and 0.5 MN (Duncan Multiple Range test). Vaccarino et al. 1985b, Copyright 1985, Springer-Verlag.

The Role of Presynaptic Dopamine Terminals in the Nucleus Accumbens in Opiate Reinforcement

Catecholamines have been strongly implicated in the reinforcing properties of psychomotor stimulants (Pickens et al. 1978). More specifically, the reinforcing properties of psychomotor stimulants have been linked to the activation of central dopamine (DA) neurons and their postsynaptic receptors. When the synthesis of catecholamines is inhibited by administering alpha-methyl-para-tyrosine, an attenuation of the reinforcing effects of psychomotor stimulants occurs (Pickens et al. 1978; Jonsson et al. 1971). Furthermore, low doses of DA antagonists will increase the response rates for intravenous injections of d-amphetamine (Davis and Smith 1975; Yokel and Wise 1975; Yokel and Wise 1976). These authors hypothesized that a partial blockade of DA receptors produced a partial blockade of the reinforcing effects of d-amphetamine. Thus, animals are thought to compensate for decreases in the magnitude of the reinforcer by increasing their self-administration behavior, as discussed above and shown in figure 2. The role of DA in the reinforcing properties of cocaine was extended by the observation that 6-hydroxydopamine (6-OHDA) lesions of the N.Acc. produced extinction-like responding and a significant and longlasting reduction in self-administration of cocaine over days (Roberts et al. 1977; Roberts et al. 1980; Lyness et al. 1979).

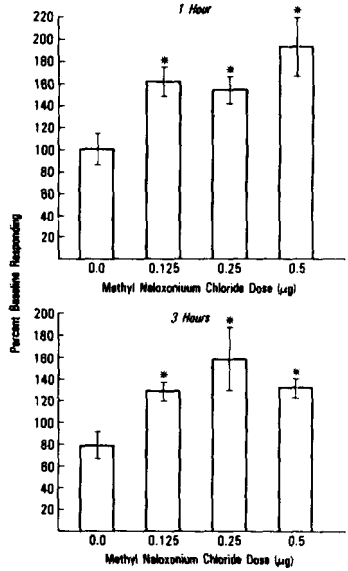

FIGURE 6. *Percent baseline (predrug day) responding for IV heroin during the first hour (top graph) and for the total 3 hours (bottom graph) of the heroin setf-administration session following MN injections into the nucleus accumbens*

NOTE: Asterisks indicate a significant difference ($p<.05$) from saline vehicle (0.0 dose) (Duncan Multiple Range test). Vaccarino et al. 1985b. Copyright 1985, Springer-Verlag.

These results demonstrated that postsynaptic blockade of DA receptors or destruction of presynaptic DA terminals in the region of the N.Acc. significantly decreased the reinforcing value of psychomotor stimulants.

Thus, the mesolimbic DA system appears to be critical for psychomotor stimulant reinforcement (Roberts et al. 1977; Roberts et al. 1980; Lyness et al. 1979), and any hypotheses regarding a role for DA in opioid reinforcement would likely focus on this same mesolimbic system. This is particularly relevant, since rats will maintain self-administration, of morphine applied directly into the brain region containing the mesolimbic DA cell bodies, the VTA (Phillips and LePiane 1980; Bozarth and Wise 1981b), as well as the N.Acc. itself (Olds 1982). To clarify further the role of mesolimbic DA neurons in opiate reinforcement, we examined the effects of DA denervation of the N.Acc. on both heroin and cocaine self-administration.

Rats were trained to self-administer intravenously heroin (0.06 mg/kg/injection) and cocaine (0.75 mg/kg/injection) for 3 hours on

alternate days. This alternating drug self-administration procedure was continued until stable intake and titration on both drugs had occurred. For each rat, each drug was delivered via a given lever (left or right), and a colored light (red or yellow) was used as a constant discriminative stimulus, which was turned on at the onset of the infusion and remained on for 20 seconds. Animals that showed stable baselines over 3 days with each drug were given an intracerebral injection of either 6-hydroxydopamine (6-OHDA) or vehicle into the N.Acc., as described previously (Joyce and Koob 1981). These lesions produced a 94 percent depletion of DA in the N.Acc. but no significant decrease in the anterior striatum (Pettit et al. 1984). Four days following the lesion, the rats were allowed to resume the alternating schedule of self-administration as described above. The 6-OHDA lesions initially produced an attenuation in both cocaine and heroin self-administration on the first self-administration trial postlesion. Subsequently, heroin responding recovered with time, gradually increasing to 76 percent of prelesion baseline levels, on average. In contrast, cocaine responding continued to decrease over trials after 6-OHDA. By the fifth trial postlesion, cocaine self-administration rates were reduced to 30 percent of prelesion baseline levels; this percent change was significantly different from that for heroin rates (Pettit et al. 1984). No significant differences in responding to cocaine or heroin postlesion were seen in sham-operated rats (Pettit et al. 1984). In three animals, heroin responses were distinctly higher than cocaine responses postlesion, demonstrating an absolute as well as a relative difference in responding (Pettit et al. 1984). These results demonstrate that selective lesions of the presynaptic DA input to the N.Acc. can significantly attenuate cocaine self-administration, without influencing heroin responding.

An important factor in this study was that the effects of each lesion could be measured on the *two* independent drug variables almost simultaneously. Thus, differential effects could not be attributed to different DA depletion levels, since for each subject the lesion had specific effects on the self-administration of cocaine and heroin, and these effects were compared within individuals to prelesion rates. Thus, these results have direct implications as to the neural substrates responsible for the reinforcing properties of both psychomotor stimulants and opioids; the results suggest that the reinforcing properties of heroin are at some point independent of the dopaminergic neural systems mediating the reinforcing properties of cocaine.

OPIATE RECEPTOR SUBTYPES AND OPIATE REINFORCEMENT

The hypothesis that specific subtypes of opiate receptors may be involved in the reinforcing properties of opiates reflects a question of significant interest to pharmacologists (Zukin and Zukin 1984)--a question difficult to address because of technical limitations. A variety of opiate drugs are self-administered by animals, including, in rats, morphine, codeine, etonitazene, meperidine, propoxyphene, as well as the mixed agonist/antagonists

butorphanol, nalbuphine, nalorphine, and pentazocine (Collins et al. 1984). The opiate antagonists cyclazocine and naloxone were inactive; ethylketazocine had clear reinforcing effects in rats (Collins et al. 1984) but not in monkeys (Woods et al. 1979). However, because of different pharmacokinetic characteristics of these drugs, any correlation of potency for self-administration with binding affinity to opiate subtype would be meaningless. An alternative approach has been to examine the reinforcing efficacy of opiate agonists more or less specific to a given opiate receptor, and from these data to generate hypotheses regarding a possible subtype of opiate receptor particularly involved in opiate reinforcement.

Evidence From Conditioned Place-Preference Studies

A number of opiate drugs, when paired as unconditioned stimuli to a particular environment, show the capability of imparting to that environment a positive reinforcing property (Mucha et al. 1982; Spyraki et al. 1983; Stolerman et al. 1978; Amalric et al., in press; Mucha and Iversen 1984). The measure of reinforcement in this paradigm is the return of an animal in a choice situation to the environment previously paired with the drug, i.e., a preference for that environment over an unpaired environment. Analogous to the classical taste preference procedures used by Wikler and associates in dependent rats (Wikler 1965), nonphysically dependent animals will show readily such a conditioned place preference with as few as one prior pairing with a drug such as heroin (Mucha et al. 1982).

In a recent study (Mucha and Herz 1985), the reinforcing efficacy of various opioid agonists acting preferentially on the kappa and mu opioid receptors was assessed using the place-preference conditioning paradigm. Kappa receptor agonists such as U50-488 and (-)bremazocine produced place aversions, whereas mu agonists such as morphine, fentanyl, and sufentanil produced place preferences (Mucha and Herz 1985).

Similarly, in our laboratory, experiments were directed at examining the reinforcing properties of beta-endorphin (B-END) using a discrete-trials conditioned place-preference test. B-END binds preferentially to mu and delta opiate receptor subtypes. The paradigm paired an intracerebroventricular injection of an opioid peptide with one distinct environment and saline with another distinctly different environment on alternate days for 6 days (training). Peptide injections were paired with the least preferred environment, based on the preference of the rats in a pretreatment session; however, there were no differences in group preference for either environment. B-END (1.5, 2.5, 5.0, or 10.0 µg/rat) was injected intracerebroventricularly (ICV 2 µl volume) immediately before the rat was placed in the training box for 30 minutes. Control rats were injected with saline, morphine (10 µg ICV), or heroin (0.5 mg/kg subcutaneously (SC)). After training, each rat was tested drug-free in a double-environment box where each end was identical to the training environments, with a

smaller, gray, neutral area in the center. Time spent in each end of the test box was recorded over a lo-minute period.

In a separate experiment, rats were similarly treated except that they received only one pairing of ICV B-END (2.5 µg) to the least preferred environment, plus SC injection of 0.04, 0.20, or 1.0 mg/kg of naloxone.

Heroin (0.5 mg/kg SC) produced strong preference for the heroin-paired environment. Rats also showed dose-dependent place preference for the environment paired with B-END (figure 7). Rats injected with the higher doses showed no preference for the paired environment, but did show catalepsy and immobility with B-END (Amalric et al., in press). Naloxone effectively blocked this place preference at a dose as low as 0.04 mg/kg (figure 8). This dose had no effect on its own, but higher doses of naloxone alone produced a place aversion (Amalric et al., in press).

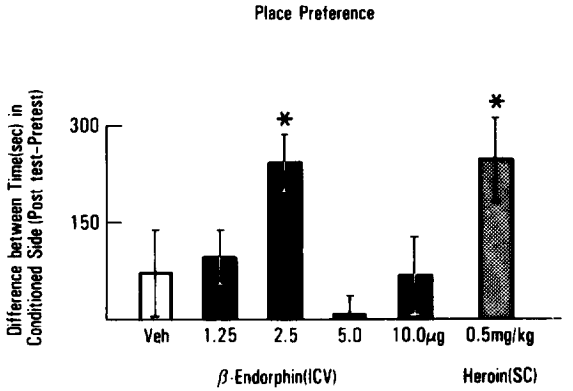

FIGURE 7. *The effects of intracerebroventricular (ICV) injections of beta-endorphin on place preference in the rat*

NOTE: Data represent mean ± S.E.M. of the difference In time between pretest and posttest on the conditioned side during a 10-minute test after 6 days of conditioning (B-END for 3 days on the paired side alternating with saline for 3 days on the unpaired side). There was no systematic preference for the white or black side prior to training. Heroin was injected SC. Asterisks indicate significant difference from saline (Student's t-test).

These results demonstrate positive reinforcing properties for B-END. Given that B-END interacts mainly with mu and delta receptors, these results, combined with the results of studies with other selective opiate agonists (Mucha and Herz 1985), suggest that mu and delta receptors are the opiate receptor subtypes important for opiate reinforcement.

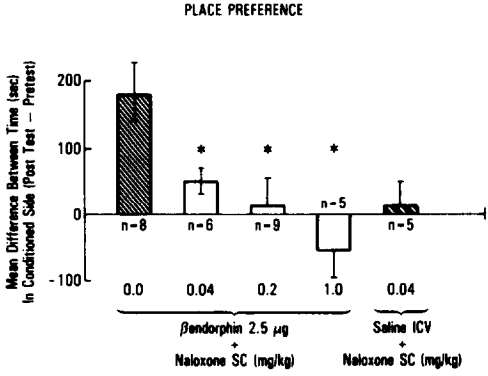

FIGURE 8. *The effects of natoxone injected systemically SC on place preference produced by ICV injection of B-END*

NOTE: Data represent mean ± S.E.M. of the dlfference in time between pretest and oosttest on the conditioned side during a 10-minute test after 2 days of conditioning. Asterisks indicate significant difference from B-END (Student's t-test).

Evidence From Self-Administration Studies

Further support for this hypothesis has been obtained using the self-administration procedure. As discussed above, many opiate agonists and mixed agonist-antagonists are self-administered by rats and monkeys. However, in rats, at least one study has found the putative kappa agonist ethylketazocine to have reinforcing properties (Collins et al. 1984). even though it does not produce place preference or aversion.

To examine this question further, rats prepared with intravenous catheters and trained to self-administer heroin were subjected to a series of acute treatments with mu and kappa agonists during self-administration. Following establishment of a dose-dependent pause in opiate self-administration, verification of the ability of a given compound to substitute for heroin was obtained by replacing the heroin with the drug in question.

Six rats were allowed to self-administer heroin for 3 hours per day on an FR 5 schedule of reinforcement. During response pause testing, the rats were allowed a 1-hour period of access to heroin; they were then removed from the apparatus and injected with 0.01, 0.02, or 0.04 mg/kg of fentanyl (mu agonist) or 0.25, 0.50, or 1.0 mg/kg of U50-488 (kappa agonist). Following each series, the respective agonists were substituted as the drug to be self-administered, at a dose deemed equivalent to that used for heroin.

As can be seen in table 1, fentanyl produced a dose-dependent pause in self-administration when injected SC during a heroin

TABLE 1. *Effects of fentanyl and U50-488 on heroin self-administration in the rat*

Compound	Response Pause (Secs)*	Compound	Self-Administration (Number Infusions)*
Fentanyl		Fentanyl	
0	10.0±1.3‡	0.006 mg/kg/inj	Total 3 hrs 15.2±2.4
0.01 mg/kg	33.7±5.9		1st hr 6.7±0.7
0.02 mg/kg	51.5±7.8		2nd hr 4.5±1.0
0.04 mg/kg	74.0±7.6		3rd hr 4.0±0.9
U50-488		U50-488	
0	10.0±1.3*	0.15 mg/kg/inj	Total 3 hrs 5.5±1.7
0.25 mg/kg	17.2±2.9		1st hr 4.5±1.4
0.50 mg/kg	36.8±15.0		2nd hr 0.5±0.5
1.00 mg/kg	13.0±2.5		3rd hr 0.5±0.3
Heroin		Heroin	
0	10.0±1.3*	0.06 mg/kg/inj	Total 3 hrs 16.0±1.9
0.1 mg/kg	21.3±6.8		1st hr 7.2±0.9
0.2 mg/kg	43.0±11.9		2nd hr 5.0±0.7
0.4 mg/kg	65.2±10.9		3rd hr 3.8±0.7
		Saline	Total 3 hrs 6.0±1.9
			1st hr 4.5±2.1
			2nd hr 0.0±0.0
			3rd hr 0.3±0.2

*Values represent mean ± S.E.M. for six rats.

‡Represents the same observations for a single systemic saline injection 1 hour into a 3-hour heroin self-administration session.

self-administration test. Fentanyl also readily substituted for heroin on a subsequent test day at a dose of 0.006 mg/kg/injection. U50-488 failed to produce any systematic response pauses at doses as high as 1.0 mg/kg and failed to substitute for heroin at a dose of 0.15 mg/kg/injection on a subsequent trial. These results suggest that the reinforcing properties of heroin are related to an activation of the mu opiate receptor subtype.

SUMMARY

The studies reported herein summarize our work to date aimed at determining the neurochemical substrates for the reinforcing properties of opiates. Rats were trained to self-administer heroin intravenously in daily 3-hour sessions, and pharmacological blockade and neurotoxin-induced lesions were used to define the neurochemical substrates for this reinforcing action. Low-dose DA receptor blockade failed to alter heroin self-administration but significantly increased cocaine self-administration, presumably reflecting a decrease in the reinforcing effectiveness of cocaine. Destruction of presynaptic DA terminals within the N.Acc. produced extinction of cocaine, but not heroin, self-administration. Opiate receptor blockade with systemic naloxone increased heroin, but not cocaine, self-administration. Methylnaloxonium injections into the N.Acc. were effective in increasing heroin self-administration at doses one-eighth those observed for intracerebroventricular injections.

Reinforcement has been explored using a place-preference procedure and a self-administration drug-substitution paradigm. Mu/delta agonists such as B-END readily produce a naloxone-reversible place preference. Fentanyl derivatives also produce place preference and substitute for heroin during self-administration. The kappa agonist U50-488 produces place aversion, not place preference, and does not readily substitute for heroin. Altogether, these results suggest that mu/delta receptor subtypes in the region of the N.Acc. may be an important neurochemical substrate for opiate reinforcement.

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Opiate Receptor Subtypes Associated With the Brain Mechanisms of Feeding and Reward

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INTRODUCTION

Determination of the opiate receptor subtypes involved in a given behavior depends, at the present time, on the ability to determine the relative potencies of various receptor agonists for the behavior in question. The critical measure of potency is the concentration needed, at the receptor, to produce the behavioral action in question. Since the defining feature of drug abuse is compulsive drug self-administration, the most relevant drug action for determining the receptor types associated with abuse would be the rewarding or reinforcing action of opioids. Unfortunately, it is very difficult to determine the necessary opfoid concentration, at the relevant receptor, for a just-detectable rewarding effect.

This difficulty arises from several facts. First, the paradigms in which reinforcing effects of drugs are identified are paradigms in which the animal, rather than the experimenter, controls the number and timing of the injections and, thus, the dose administered. Second, the concentration at the relevant receptor is not simply proportional to the dose administered. Even if all animals self-administered the same amount of a drug at the same intervals, dose at the relevant receptor would be difficult to determine for different opioids. If the drugs were given intravenously, differences in ability to penetrate the brain, as well as differences in rate of metabolism, would have to be considered for each compound. If the drugs were given centrally, rate of metabolism and unpredictable dosing would still be major factors, and concentration gradients between the injection site and the site of action would have to be determined for each compound. This would be a problem, as it is not yet clear how many sites of rewarding opiate action there are in the brain, and for neither of the two current candidate sites are the boundaries of the population of target neurons known. Another problem is that in order to have good control over concentration of the drug at the receptor, it would be preferred that long-acting agents be used so that concentration did not vary significantly over time; this would conflict with the needs of the behavioral paradigm, where short-acting agents are

preferred because they increase the number of responses and, thus, increase the precision of response-rate estimates.

The best paradigm for determining opioid receptor involvement in behavior would involve experimenter, rather than animal, controlled dosing and long-acting, rather than short-acting, drugs. These conditions are compatible with studies of independent behaviors, such as feeding, but not with drug self-administration itself. There is evidence, however, to suggest that the same brain mechanisms may be involved in feeding and opiate self-administration. To the degree that this evidence is credible, certain feeding paradigms may thus serve as models that predict the relative rewarding potency of various opioid compounds. This chapter summarizes our recent explorations of this possibility.

The evidence linking the mechanisms of reward and feeding is, for the most part, evidence from brain stimulation studies. Stimulation of the lateral hypothalamic medial forebrain bundle can be rewarding, and stimulation at the same brain loci can induce feeding and other biologically primitive, species-typical behaviors (Glickman and Schiff 1967). In general, drugs and treatments that influence stimulation-induced feeding similarly influence intracranial self-stimulation (Hoebel 1969). Moreover, the refractory periods for the populations of neurons underlying each of the two behaviors have been characterized and found to be similar (Hawkins et al. 1983). We have redone the early estimates of refractory period distribution using a finer-grained analysis, and we have found that at least two subpopulations of medial forebrain fibers are implicated in brain stimulation reward: a very fast, atropine-sensitive subpopulation, and a slower, atropine-insensitive subpopulation (Gratton and Wise 1985a). We have extended the fine-grained analysis to stimulation-induced feeding, and we found evidence that the same subpopulations participate in this behavior as well (Gratton and Wise 1985b). Moreover, when the dorsal-ventral extent of the system is mapped using a moveable electrode, each electrode movement that causes an increase in the reward threshold also causes an increase in the feeding threshold (figure 1).

The circuitry underlying brain stimulation reward and stimulation-induced feeding is not understood in detail, but the current view is that lateral hypothalamic stimulation activates descending fibers (Shizgal et al. 1980), which transsynaptically activate ventral tegmental area (VTA) dopamine cells (Wise and Bozarth 1984). Morphine is known to interact with this circuitry at the level of the VTA dopaminergic cell bodies (Matthews and German 1984; Ostrowski et al. 1982), where it produces direct rewarding effects (Bozarth and Wise 1981; Phillips and LePiane 1980) and it facilitates both brain stimulation reward (Broekkamp et al. 1976) and stimulation-induced feeding (Wise et al. 1985). Naloxone does not block but attenuates both behaviors (Carr and Simon 1983; Stapleton et al. 1979). VTA morphine injections also facilitate free feeding; naloxone blocks this effect and inhibits feeding in

morphine-free animals (Wise et al. 1985). Morphine injections into the periaqueductal gray (PAG) substance, on the other hand, inhibit self-stimulation (Broekkamp et al. 1976), stimulation-induced feeding (Wise et al. 1985), and free feeding (Wise and Raptis, unpublished observations).

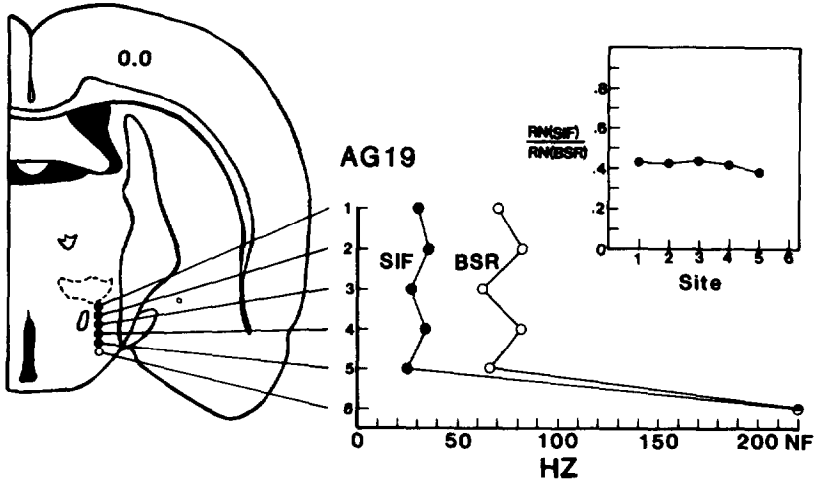

FIGURE 1. *Variations in brain stimulation reward (BSR) and stimulation-induced feeding (SIF) threshold as a function of electrode placement fop rat AG19*

NOTE: The absolute values for the two behaviors differ because of task variables (the BSR thresholds would be lower than the SIF thresholds if the animals could use nose-poke, rather than lever-press, stimulation). The ratio of the SIF threshold to the BSR threshold was constant, however, as shown in the Inset.

Thus, it appears that the mesolimbic dopaminergic fibers play a role in both reward and feeding, and that opiates can activate these fibers at the level of the VTA cell bodies. These facts suggest that the effects of various opioids on stimulation-induced feeding--of considerable interest in its own right--may provide a useful model from which to predict the relative potency of the opioids in reward function. We have studied the relative potency of VTA and PAG injections of three opioids in the stimulation-induced feeding paradigm.

METHODS

Rats with lateral hypothalamic electrodes were tested for stimulation-induced feeding in a 25x25 cm box with 45 mg Noyes food pellets covering the floor. Cathodal pulses *of* 0.1 ms duration were administered at a frequency of 50 Hz, and, in animals that ate under stimulation, stimulation intensity was adjusted to produce the eating of just three pellets in 10 seconds

time. Time to eat three pellets was then determined over the range of effective stimulation frequencies. For these tests, stimulation was administered in 20-s trains with 20-s intertrain intervals; no eating was ever seen in the intertrain intervals, as the animals were allowed to satiate on the test food just prior to testing. Four blocks of stimulation tests were given each day: stimulation frequency was increased, from ineffective levels to maximally effective levels, in the first and third blocks and was decreased, from maximally effective levels to ineffective levels, in the second and fourth blocks. The animals were tested daily in this fashion until day-to-day fluctuations in their frequency-latency functions were minimal; then drug testing began.

Morphine was chosen as a prototypic mu agonist, U-50,488H as a specific kappa agonist (Vonvoigtlander et al. 1983), and D-Pen2, D-Pen5 enkephalin as a specific delta opioid agonist (Mosberg et al. 1983). These drugs were microinjected into the VTA or the PAG by way of chronically implanted guide cannulae (o.d.=0.4 mm). Microinjections were carried out by inserting into the guide cannula an injection cannula (o.d.=0.28 mm) connected by polyethylene tubing to a 1 µl Hamilton microsyringe. The injection cannula extended beyond the tip of the guide by 1 mm; the injector was left in place for 2 minutes after the injection was complete. Injection volume was 0.25 µl and doses of 8 and 16 nmoles were infused in 30 seconds into VTA or the PAG on separate days. We have previously shown that such doses of morphine into VTA facilitate stimulation-induced eating, while such injections into PAG inhibit it (Wise et al. 1985). Each animal was tested alternately with VTA then PAG microinjections such that a minimum of 1 week elapsed between two consecutive microinjections into the same brain site. Testing began 20 minutes after each microinjection and was otherwise similar to the tests made under no-drug conditions.

RESULTS

VTA microinjections facilitated stimulation-induced eating, whether morphine, U-50,488H or D-Pen2,D-Pen5 enkephalin was infused. Time to complete eating of three food pellets decreased, as did the minimum stimulation frequency required to induce eating at a given latency. The three agents were similar in potency (figure 2), although D-Pen2,D-Pen5 enkephalin appeared more effective in inducing locomotor activity. Morphine and U-50,488H were as effective in the third and fourth test blocks as in the first and second; D-Pen2,D-Pen5 enkephalin appeared to lose effectiveness by the third and fourth blocks of tests.

PAG injections of morphine inhibited stimulation-induced eating (figure 3). The other two agonists, however, produced no effects in the PAG. The threshold dose for inhibition of stimulation-induced eating by morphine in the PAG was about twice the threshold dose for facilitation of stimulation-induced eating by morphine in the VTA.

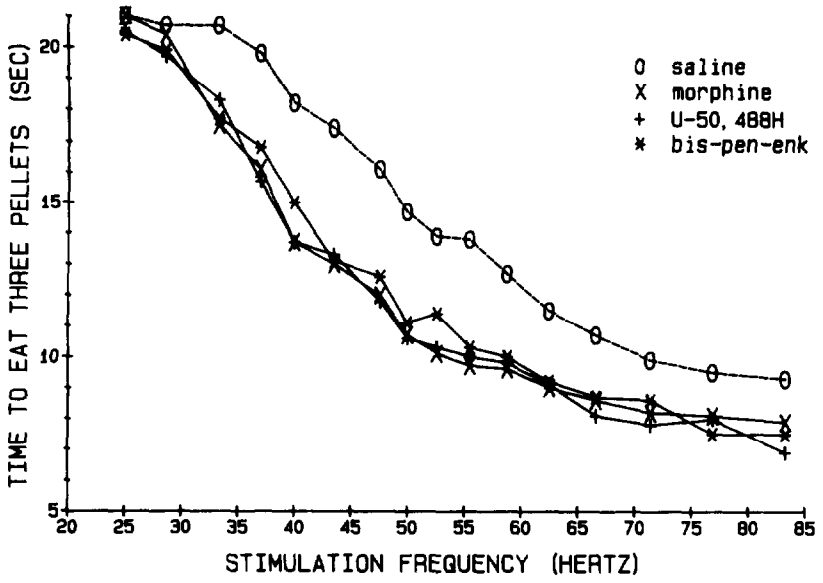

FIGURE 2. *Effects of ventral tegmental injections of selective mu (morphine), kappa (U-50,488H), and delta (bis-pen-enk) opioid receptor agonists on stimulation-induced feeding*

NOTE: The same injection dose of 8 nmoles was used for each agent; injectIon volume was 0.25 µl. Mean data from four animals are shown.

DISCUSSION

These data suggest opioid interactions with the VTA mechanisms involved in feeding, or in the facilitation of feeding, and with PAG mechanisms that inhibit or conflict with feeding. While the drugs used are relatively selective for mu, kappa, and delta opioid receptors, their equal potency in the VTA tests does not mean that all three receptors are involved in the mechanism of this behavior. Since equal injection doses do not guarantee equal concentrations at the relevant receptors, it is possible that one of the agents was more potent--at the critical receptors--than were the other two. Thus, while mu, delta, and kappa receptors might all be linked to the VTA mechanism of opioid-induced facilitation of stimulation-induced feeding, it is also possible that only one or two of these receptors is involved and that *one or* more of the agonists had its action at a nonpreferred receptor.

Clearly, not all the agonists are acting on the same class of receptor. The fact that neither the delta agonist nor the kappa agonist had morphine-like effects in the PAG suggests that neither the kappa nor the delta agonist was acting on the same class of mu

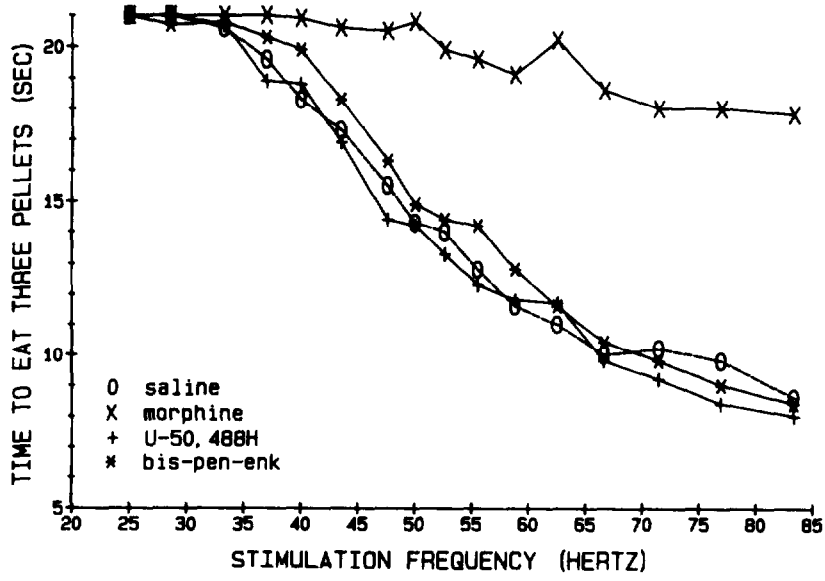

FIGURE 3. *Effects of periaqueductal grey injections of selective mu (morphine), kappa (U-50,488H), and delta (bis-pen-enk) opioid receptor agonists on stimulation-induced feeding*

NOTE: The ssme injection dose of 16 moles was used for each agent; injection volume was 0.25 µl. Mean data from four animals are shown.

receptor as was involved in the PAG effects. Further tests are needed to narrow the possibilities in this regard. Nonetheless, the fact that U-50,488H and D-Pen2,D-Pen5 enkephalin had different effects, relative to morphine, at different sites indicates at least some degree of behavioral selectivity of the agonists and some degree of specificity of the receptors in the facilitation and inhibition of feeding.

The opposite effects of PAG and VTA morphine on stimulation-induced feeding parallels the effects of these injections on brain stimulation reward (Broekkamp et al. 1976) and strengthens the evidence for a common mechanism for stimulation-induced feeding and brain stimulation reward. The effects of the kappa and delta agonists on brain stimulation reward have not yet been tested. The hypothesis that stimulation-induced feeding predicts the rewarding effects of various drug manipulations would indicate that VTA injections of U-50,488H and D-Pen2,D-Pen5 enkephalin should both facilitate brain stimulation reward and produce rewarding effects in their own right. Tests of this hypothesis are under way.

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In Vivo Interactions Among Opiate Receptor Agonists and Antagonists

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INTRODUCTION

It is a never-ending quest by medicinal chemists and pharmacologists to synthesize ligands that have selective actions at known receptor types and subtypes. Often, newly discovered agonist and antagonist ligands are initially heralded as being "pure" and "selective." Over time, as the pharmacological profiles of these compounds become more thoroughly characterized, these descriptions become meaningless as biochemical and pharmacological data establish that the ligands "cross-react" with different receptors, or function as mixed agonist and/or antagonist molecules. Based upon evidence with biologically derived substances, this may not be surprising. For example, although epinephrine has a preponderant effect upon ß adrenergic receptors and norepinephrine acts predominantly upon α adrenergic receptors, it is well known that given in adequate concentrations, these ligands will cross-react with both receptor systems (Goldstein et al. 1974). Furthermore, adrenergic "agonist" drugs with actions at α_2 receptors (e.g., clonidine) may serve to antagonize endogenous α agonist actions, presumably by activating presynaptic receptors which decrease norepinephrine release (Goodman and Gilman 1975).

The cross-reactivities and differences in intrinsic activities among agonist and antagonist ligands are fundamental concerns of pharmacologists. Several in vivo and in vitro methods have been used to identify the pharmacologic profiles of various agonist and antagonist ligands. Prominent among these are various receptor binding assays, which have been used extensively to identify ligand selectivity. However, receptor binding studies alone provide little or no evidence of "intrinsic activity," a phenomenon that requires the measurement of biological responses following ligand-receptor interactions. It is generally assumed that agonists have intrinsic activity, while antagonists lack intrinsic activity (Goldstein et al. 1974). One plausible explanation for this difference between agonist and antagonist ligands is that they bind in a different manner to receptors.

Over the past few years, we have become interested in studying apparent "cross-reactivities" among opioid responses using various physiological, pharmacological , and neurochemical measurements. These in vivo experiments have used specific combinations of opioid agonist and antagonist ligands to reveal consistent patterns of responses. Using three experimental paradigms, including the rat endotoxic shock model, the rat flurothyl seizure model, and in vivo changes in striatal cyclic adenosine monophosphate (cAMP), we provide evidence indicating that μ antagonists (some of which share κ agonist properties) also block the effects of δ antagonists. Our working hypothesis is that these repeating patterns of opioid cross-reactivities reinforce other evidence suggesting that there apparently are interactions among the various subtypes of opiate receptors (Lee and Smith 1980; Bowen et al. 1981; Rothman and Westfall 1982; Vaught et al. 1982). We and other researchers postulate that these interactions may occur via allosteric mechanisms or may be mediated by interconversions of a common receptor macromolecule.

MULTIPLE OPIATE RECEPTORS

Several subtypes of opiate receptors have been characterized over the last decade. Thus, many laboratories have presented evidence to indicate that these receptors exist as separate macromolecular species with anatomically distinct distributions and unique pharmacological profiles (Holaday and Tortella 1984; Paterson et al. 1983). Moreover, sequential administration of opioid agonist ligands has provided evidence for a lack of cross-tolerance among the μ, δ, and κ receptor subtypes (Schulz et al. 1980; Schulz et al. 1981; Vonvoigtlander et al. 1983). Indeed, these observations may be considered as strong evidence for the individuality of multiple opiate receptors. Additionally, cross-protection studies have provided further evidence to distinguish these receptors as separate entities (Goldstein and James 1984; Robson and Kosterlitz 1979).

Despite the strong case that can be made for separating the various subtypes of opioid receptors , contrasting evidence has also accumulated to support the concept that opioid receptor subtypes may not be physically or functionally independent. Several investigators have-performed in vivo and in vitro experiments to indicate that μ and δ receptor sites may functionally interconvert (Bowen et al. 1981) or interact through noncompetitive molecular mechanisms (Lee and Smith 1980; Rothman and Westfall 1982; Vaught et al. 1982). Consistent with these observations, evidence from our laboratories also indicates that interactions among μ and δ binding sites may occur in vivo (D'Amato and Holaday 1984; Holaday and D'Amato 1983; Holaday et al. 1983; Tortella and Holaday 1984; Tortella et al. 1985a; Tortella et al. 1985b).

As reviewed above, it is probable that agonist and antagonist ligands bind in a different manner to their respective binding sites on receptors. Thus, one way to demonstrate the presence or absence of interactions among opiate binding sites is to evaluate

responses following administration of combinations of agonist and antagonist ligands. Using arterial pressure responses in endotoxic shock, flurothyl seizure thresholds, and in vivo changes in striatal cAMP as three different experimental endpoints, we have observed that certain μ antagonists (which may also demonstrate κ agonist effects) prevent δ antagonists from blocking or reversing δ agonist effects. This concept of "antagonists of antagonists," first mentioned by Villarreal and colleagues (Villarreal et al. 1982; Herrera et al. 1982) for opiate systems, has also been described for ligand interactions at the benzodiazepine receptor (Schweri et al. 1982; Ninan et al. 1982). Specifically, ß-carbolines, the "active" or "inverse" agonists at this receptor, administered at doses which do not show intrinsic activity, block the antagonistic action of the antagonist Ro 15-1788 against benzodiazepines. Similarly, in our studies with opioid ligands summarized below, we observe a consistent pharmacological profile indicating that certain μ antagonists also block the antagonistic actions of δ antagonists. Based upon these concepts of agonist-antagonist interactions, a new perspective on the separability of opioid receptor subtypes may be gleaned.

Endotoxic Shock

Over the past several years, we have demonstrated that the activation of endogenous opioid systems by circulatory shock or CNS ischemia contributes to the pathophysiology of these ischemic disorders (Holaday 1983; Holaday 1985). Specifically, treatment with the opiate antagonist naloxone results in a significant improvement in cardiovascular performance, metabolic status, neurologic function, and survival in many basic and clinical studies of shock and CNS ischemia (Holaday 1985). Because of the duality of action of endogenous opioids upon cardiovascular and analgesic systems, administration of naloxone for the treatment of shock or CNS injury may also enhance pain by antagonizing endogenous or exogenous opioid analgesia, However, if ischemia and analgesia are mediated by different types of opiate receptors, the use of "selective" opiate receptor antagonists should provide for a dissociation of these effects. Naloxone, which is generally accepted as a "pure" opiate antagonist, lacks adequate selectivity for the different opiate receptor subtypes. Thus, our initial investigations into the type(s) of opiate receptors responsible for ischemia and analgesia used more "selective" lfgands and resulted in the unique pharmacological observations described below.

In order to define the opioid receptor subtype responsible for the hypotensive effects of endotoxic shock, we initially used the μ antagonist naloxazone. Naloxazone (now known to be active following biochemical conversion to naloxonazine) is reported to be a longlasting alkylator of μ-antagonist sites (Holaday et al. 1983). In later experiments, we used ß-funaltrexamine (ß-FNA), a different μ antagonist alkylator defined by Portoghese, Takemori, and

colleagues (Takemori et al. 1981; Ward et al. 1982). To achieve an antagonism of δ sites, we used the δ-selective antagonist ICI 154,129 or high doses of naloxone (D'Amato and Holaday 1984; Holaday and D'Amato 1983; Holaday et al. 1983).

We demonstrated that the 6 antagonist ICI 154,129 or high doses of naloxone successfully reversed endotoxic shock hypotension, whereas the μ antagonists naloxazone or ß-FNA failed to do so (Holaday et al. 1983; D'Amato and Holaday 1984). Conversely, the δ antagonist ICI 154,129 failed to alter morphine (u-induced) antinociception, whereas naloxazone and ß-FNA blocked morphine antinociception (D'Amato and Holaday 1984). From these results, it appears that endogenous opioids released in endotoxic shock act upon δ (not μ) receptors to produce their pathophysiological effects. However, these studies also revealed an unexpected finding; if naloxazone or ß-FNA were administered prior to the ICI 154,129 or naloxone in rats subjected to endotoxemfa, the latter substances were no longer effective in reversing endotoxic shock hypotension (D'Amato and Holaday 1984; Holaday and O'Amato 1983; Holaday et al. 1983). This concept is schematically represented in figure 1.

ß-FNA exhibits κ agonist properties in addition to its irreversible antagonism of the μ site (Ward et al. 1982). It thus has a pharmacological profile like that of the peptide dynorphin 1-13, an opioid compound known for its κ agonist actions, but which also has persistent μ antagonist properties (Garzon et al. 1982). Not surprisingly, dynorphin 1-13 produced results similar to our earlier findings with naloxazone and ß-FNA. Pretreatment with dynorphin 1-13 had no effect by itself in altering endotoxic hypotension, yet this pretreatment significantly antagonized the usual therapeutic effects of naloxone in this model (Holaday et al. 1984).

More recently, we have used the new s-selective antagonist, ICI 174,864 (Cotton et al. 1984) alone and in combination with dynorphin pretreatment (Long et al. 1984). As expected, ICI 174,864 reversed endotoxic shock hypotension. Moreover, as observed with dynorphin-naloxone studies (Holaday et al. 1984), pretreatment with 1.0 mg/kg dynorphin 1-13 intravenously (IV) 2 hours prior to endotoxin injection also prevented the therapeutic effects of ICI 174,864 (Long et al. 1984) (table 1).

From these studies of endotoxic shock in rats, we concluded: (1) endogenous opioids contribute to endotoxic hypotension by acting on δ opiate receptors, (2) dynorphin peptides may exert actions well beyond those predicted from metabolic half-lives obtained in tissues, and (3) compounds such as naloxazone, ß-FNA, and dynorphin act as μ antagonists which also antagonize the actions of δ antagonists (figure 1).

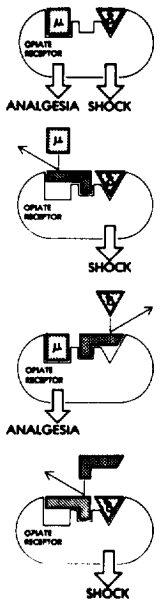

FIGURE 1. *Schematic representation of possible allosteric interactions among μ and δ binding sites on the opioid receptor complex*

NOTE: TOP: Morphine analgesia Is represented by μ in the square, whereas circulatory shock, presumably resulting from endogenous δ ligand activation, is represented by the δ in the triangle. TOP MIDDLE: The putative μ antagonists ß-FNA and naloxone (represented by 'L' shape pointing left) blocked analgesic responses to the μ agonist morphine while having no effect upon shock. BOTTOM MIDDLE: By contrast, ICI 154,129, representing a δ antagonist ('L' shape pointing right), was without effect upon morphine (μ) analgesia while reversing shock hypotension. BOTTOM: However. prior treatment with the irreversible μ antagonists (bottom of schematic) prevented the actions of the δ antagonist in reversing shock, indicating possible allosteric interactions between these opioid binding sites.

Flurothyl Seizures

To confirm the pattern of opioid interactions initially observed in the endotoxic shock model, we investigated the same interactions using a model of flurothyl-induced seizures in rats (Tortella and Holaday 1984). Flurothyl is a volatile convulsant, and rats exposed to flurothyl experience clonic convulsions within approximately 360 seconds (Cowan et al. 1979; Tortella et al. 1981). Unlike the endotoxic shock model where endogenous opioids are physiologically released to act upon δ receptors, in the flurothyl seizure studies, opioid agonists and antagonists were pharmacologically administered. Using this approach, we and others have shown that several classes of opioid agonist ligands

TABLE 1. *Effects of dynorphin pretreatment on reversal of endotoxic hypotension by the δ antagonist ICI 174,864*

Population	Pretreatment (1st Injection)	Postshock Treatment (2nd Injection)	Increase in MAP 15 Min After 2nd Injection
15	saline	saline	3.0±3.8
13	saline	ICI 174,864[2]	17.1±4.2*
7	dynorphin 1-13[3]	saline	2.3±5.7
11	dynorphin 1-13	ICI 174,864	5.6±3.2**

[1]Following a fall in mean arterial pressure (MAP) of 20 mm Hg produced by endotoxin (22.5 mg/kg IV).

[2]ICI 174,864 (3.0 mg/kg IV) was dissolved in 0.1 M Na Bicarb and injected IV.

[3]Dynorphin 1-13 (1.0 mg/kg) was dissolved in saline and injected IV.

*p<0.05 compared to saline alone.

*p<0.05 compared to saline pretreatment.

SOURCE: From Long et al. (1984).

may act upon separate receptor types to elevate seizure thresholds in this model (Cowan et al. 1979; Tortella et al. 1983). For example, the anticonvulsant effects of etorphine were completely blocked by the μ antagonist ß-FNA, whereas ß-FNA only partially antagonized the effects of the δ agonist, D-Ala^2-D-Leu^5-enkephalin (DADL) (figures 2 and 3) (Tortella et al. 1985a). Likewise, the δ antagonist ICI 154,129 completely blocked the anticonvulsant effects of DADL but was without effect on the μ actions of etorphine (Tortella and Holaday 1984; Tortella et al. 1983). Once again, consistent with the pattern of interactions observed in the endotoxic shock model, ß-FNA pretreatment prevented ICI 154,129 from further blocking the anticonvulsant effect of DADL (figure 4) (Tortella and Holaday 1984; Tortella et al. 1985a).

Additional studies provide support for these unique interactions and further demonstrate that dynorphin 1-13 exerts longlasting μ antagonism. After the initial nonopioid anticonvulsant effects of dynorphin 1-13 pretreatment have ended (2 hours) (Tortella et al. 1985b), the μ agonist DAGO (Tyr-D-Ala-Gly-NMe-Phe-Gly-ol) failed to demonstrate its usual potent anticonvulsant effects (unpublished observation). Thus, as others have shown using receptor binding assays and pharmacological responses (Garzon et al. 1982), dynorphin 1-13 is a longlasting μ antagonist. Furthermore, following pretreatment with dynorphin 1-13, the anticonvulsant

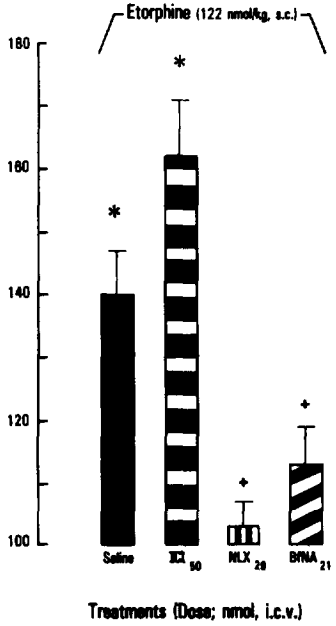

FIGURE 2. *The effect of ICI 154,129 (horizontally hatched bar), naloxone (vertically hatched bar), or β-FNA (diagonally hatched bar) to attenuate the anticonvulsant action of etorphine (solid bar) in the rat*

*Significantly different from saline control group, $p<0.01$.

†Significantly different from the 122-nmol/kg etorphine group, $p<0.05$ (Student's test).

NOTE: Subscripts represent the dose given intracerebroventricularly (ICV).

SOURCE: From Tortella et al. (1985a).

effects of subsequent DADL injections persist and can no longer be blocked with the δ antagonist ICI 154,129 or high doses of naloxone (table 2). These observations with dynorphin 1-13 confirm evidence reviewed above with endotoxic shock and flurothyl seizure models indicating that molecules with μ antagonist properties antagonize the actions of δ antagonists.

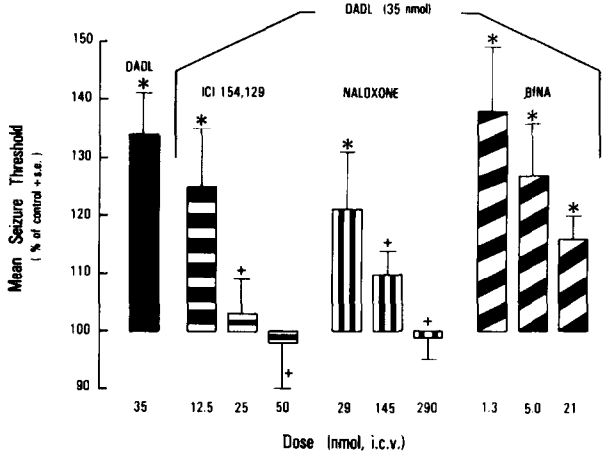

FIGURE 3. *Dose-related antagonism of the anticonvulsant effect of DADL (solid bar) in the rat*

*Significantly different from saline control group, $p<0.05$.

†Significantly different from the 35-nmol DADL group, $p<0.01$ (Dunnett's test for multiple comparisons).

NOTE: LEFT: Antagonism by ICI 154,129. MIDDLE: Antagonism by naloxone. RIGHT: Antagonism by ß-FNA.

SOURCE: From Tortella et al. (1985a).

Striatal cAMP

Opioid peptides inhibit striatal adenylate cyclase, resulting in a decrease in striatal cAMP concentrations (Childers, this volume). Evidence indicates that opioids accomplish this action by activation of the coupling Ni GTPase, resulting in an inhibition of the adenylate cyclase enzyme (Abood et al. 1985). Changes in striatal cAMP serve as a postreceptor endpoint which may serve to localize the mechanism of ligand interactions to events which precede second messenger systems. In order to confirm and refine the pattern of ligand interactions found in the in vivo experiments described above, but using a neurochemical correlate, we investigated the effects of sequential in vivo opioid antagonist and/or agonist injections on striatal cAMP levels.

Using the pharmacological strategy outlined above for the flurothyl experiments, we attempted to block the inhibitory effects of the δ agonist DADL on cAMP levels using ICI 154,129. Additionally, DADL challenge (following ß-FNA Pretreatment with or without ICI 154,129 injections) was used to determine whether or not the δ antagonist effects of ICI 154,129 would be blocked by the μ antagonist ß-FNA. As before, ß-FNA (or saline) were admin stered 18

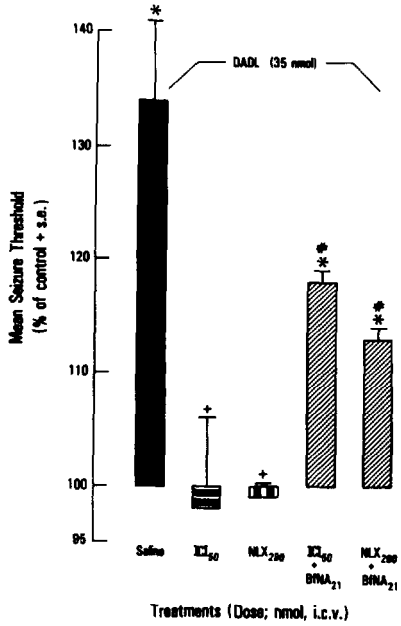

FIGURE 4. *The effect of prior treatment with ß-FNA (diagonal bars) to diminish the abilities of either ICI 154,129 (horizontal hatching) or naloxone (vertical hatching) to antagonize the anticonvulsant action of DADL (solid bar)*

*Signoficantly different from saline control, $p<0.05$.

†Significantly different from the 35-nmol OADL group, $p<0.05$ (Dunnett's test).

Significantly different from ICI_{50} †DADL and NLX_{290} †DADL groups, respectively, $p<0.05$ (Student's test).

NOTE: Subscripts represent the dose given ICV.

SOURCE: From Tortella et al. (1985a).

hours prior to ICV injections of ICI 154,129 (or saline), and DADL was injected ICV 10 minutes later. Following microwave fixation of rat brains at 20 minutes after DADL or saline challenge, brains minus cerebella were frozen for measurement of striatal cAMP levels by radioimmunoassay.

As previously reported (Childers, this volume), the δ agonist OADL reduced striatal cAMP by about 50 percent. This effect was blocked by pretreatment with ICI 154,129 or high doses of naloxone (table 3). Consistent with both the endotoxic shock and flurothyl seizure threshold studies, preliminary evidence indicates that pretreatment with the μu antagonist ß-FNA blocked the ability of the δ antagonist ICI 154,129 to antagonize the actions of the δ agonist DADL (table 3). In other words, in rats receiving

TABLE 2. *The effects of dynorphin on the delta antagonist actions of ICI 154,129 and naloxone*

Population	Treatment (µg ICV)[1] Antagonist	Agonist	$\bar{x}$ Change in Seizure Threshold (% Control ± SEM)
1 0	saline	saline	100
8	saline	DADL[2] (20)	157±13
8	ICI[3] (25)	DADL (20)	120±8*
8	naloxone (100)	DADL (2*)	106±6"
8	dyn[4] (25)	DADL (20)	151±9
8	dyn (25) + ICI (50)	DADL (20)	166±9**
8	dyn (25) + naloxone (100)	DADL (20)	132±6**

[1]ICI and naloxone were given 10 minutes prior to DADL; dyn was given 2 hours prior to OADL.

[2]DADL is D-Ala[3]-D-Leu[5]-enkephalin.

[3]ICI is the δ antagonist ICI 154,129.

[4]Dyn Is dynorphin 1-13.

*p<O.O5 compared to saline + DADL.

**p<O.O5 compared to ICI t DADL and naloxone + DADL groups, respectively.

SOURCE: From Tortella et al. (1985b).

TABLE 3. *Preliminary evidence for changes in striatal cAMP levels in rats receiving combinations of opioid agonists and/or antagonists*

Population	Treatment (µg ICV)[1] Antagonist	Agonist	pmoles cAMP/mg Wet Weight	% Control
23	saline	saline	0.48	100%
12	saline	DADL (20)	0.23	52%
7	ICI (50)	saline	0.50	104%
9	ICI (50)	DADL (20)	0.50	104%
9	ß-FNA (9.8)	saline	0.51	106%
8	ß-FNA (9.8)	DADL (20)	0.52	106%
7	ß-FNA (9.8) + ICI (50)	DADL (20)	0.27	47%

[1]ß-FNA was given 18 hours earlier, and ICI 154,129 was given 10 minutes before challenge with agonists or saline.

NOTE: These data represent preliminary results; more thorough dose response studies using more "selective" δ ligands are under way.

combined pretreatment with the opiate antagonists ß-FNA plus ICI 154,129 (each of which blocked the decrease in cAMP produced by DADL when given alone), the decrease in cAMP produced by DADL was no longer observed. Interestingly, in this model and unlike

earlier studies, ß-FNA pretreatment also blocked the ability of DADL to decrease striatal cAMP, indicating that DADL may be acting in part through μ receptors in the striatum.

Although still limited by attempts to define potential receptor interactions using in vivo approaches, data obtained from striatal cAMP studies provide an important neurochemical confirmation of earlier pharmacological data and indicate that patterns of interactions occur via mechanisms preceding second messenger systems.

SUMMARY AND CONCLUSIONS

The pattern of ligand interactions described here is consistent across three separate models of opioid actions. In each case, it was shown that three different μ antagonists (naloxazone, ß-FNA, and dynorphin 1-13) each blocked the actions of δ antagonists (ICI 154,129 or ICI 174,864).

In addition to our demonstrations that μ antagonists are also able to block the actions of δ antagonists, it is also known that many κ agonists (nalorphine, nalbuphine, bremazocine, and others) also act as μ antagonists (Gillan et al. 1981; Robson and Kosterlitz 1979; Vonvoigtlander et al. 1983). As reviewed above, our fluorothyl seizure data confirm that certain opioids (ß-FNA and dynorphin 1-13), classified as κ agonists in other systems, also demonstrate μ antagonist effects (Garzon et al. 1982; Ward et al. 1982). It is tempting to speculate that the frequent occurrence of ligands with both κ agonist and μ antagonist properties indicates that interactions among opioids are not restricted to μ and δ ligands, but may also involve κ ligands. Recently, Demoliou-Mason and Barnard (1986), using receptor binding studies, have provided evidence for allosteric interactions between κ and δ receptors. At present, we are evaluating the possibility that more selective κ agonists (e.g., U-50,488) may also act as μ antagonists and/or antagonists of δ antagonists.

To clarify the reasons underlying these sometimes confusing and interdependent phenomena of "cross reactivity," several working hypotheses may be proposed: (1) one ligand may display several moieties that account for cross-reactivity among receptor types; (2) the complex effects of ligands apparent from in vivo studies or in vitro tissue preparations must be interpreted in view of the potential involvement of networks of neurons with different opioid receptor subtypes; (3) combinations of ligands may alter the uptake or metabolism of other ligands; (4) combinations of ligands may result in alterations of receptor coupling to second messengers, and (5) cooperativity or allosteric coupling may exist among multiple binding sites of a macromolecular receptor complex. Although each emphasizes a different perspective, these multiple working hypotheses are not all mutually exclusive.

The mechanism(s) that account for the pattern of responses we observed can be considered according to the five hypotheses posed above. (1) It appears unlikely that molecules as different as the

nonpeptides naloxazone and ß-FNA would all share similar moieties with the peptide dynorphin 1-13 to explain their actions as both μv antagonists and antagonists of δ antagonists. (2) Although neuronal networks and altered neuronal uptake may still exist within the striatum, evidence obtained in our preliminary studies of striatal cAMP are consistent with the hypothesis that these pharmacological profiles are mediated by events that precede second messenger systems, possibly at the level of receptors. However, these data do not exclude the potential involvement of neuronal networks with different opioid receptor subtypes, since drugs were administered in vivo. (3) Alterations of metabolism are difficult to invoke due to the similar pharmacological profiles, yet entirely different chemical structures of the nonpeptide and peptide ligands were used. Furthermore, these actions of naloxazone, ß-FNA, and dynorphin occur at a time when these ligands do not express an intrinsic activity of their own. (4) If receptor coupling to second messengers is involved, the striatal cAMP data indicate that these interactions precede second messenger events. (5) Although by no means proven from the evidence presented in this monograph, we believe that a single agonist or antagonist ligand, acting through its binding site, may affect the binding of other agonist or antagonist ligands at other opioid binding sites by allosteric interactions (De Lean et al. 1979). Regardless of the mechanisms involved, this consistent pattern must have biological meaning.

Another noteworthy finding from these studies is that all three μ antagonists had very persistent effects. Naloxazone and ß-FNA are known to alkylate the receptor, a finding that has been reported previously. However, dynorphin 1-13, which is not described as an alkylating agent, has effects which ersist far beyond its limited plasma half-life (Garzon et al. 1982.) Furthermore, attempts to overcome the antagonism of δ antagonists produced by μ antagonists using extremely high concentrations of naloxone or the ICI compounds have been unsuccessful in the endotoxic shock studies (unpublished data). Thus, the pattern of these interactions suggest that all three substances share a noncompetitive, persistent biological effect.

Many additional studies will be required to illuminate the mechanisms of action that account for the data reviewed in this monograph. We are attempting to extend these studies with direct receptor binding experiments on rat brains following the in vivo administration of these ligands. Additionally, we are investigating the responses using more recently available κ agonists e.g., U-50,488) and δ agonists (e.g., the penicillamine derivatives).

What is the functional significance of these findings? We suggest that the classically accepted independence of multiple opioid receptor subtypes must be reconsidered. Although evidence has been presented to suggest that at least μ and δ receptors are differentially distributed within the brain (Goodman et al. 1980), others

have shown that μ and δ binding sites may represent interconvertible components of low-affinity binding (Bowen et al. 1981). Additionally, it has been demonstrated, using whole tissue target size analysis (radiation inactivation), that κ, μ, and δ "receptors" all have about the same molecular weight (approximately 90,000 Daltons) (Ott et al. 1983). Of course, biochemical analysis of extracted and partially purified receptors in various laboratories reveals differences in molecular weights (Gioannini et al. 1985). However, in the biological milieu, these opiate "receptors" may exist as collaborative complexes, much as those described for the α, β, and λ subunits of the nicotinic-cholinergic receptor.

Thus, anatomical, biochemical, behavioral, and physiological evidence is available to indicate that multiple opioid "receptors" may instead be multiple opioid "binding sites" which interact as part of the same macromolecular cluster or complex.

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