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THE OPIATE RECEPTORS

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INTRODUCTION

The extract of the opium poppy is among the oldest materials used for medicinal purposes, and the study of the major alkaloid in this extract, morphine, is one of the oldest areas of biological research. Our lack of understanding on the molecular level of how morphine and its analogues produce their effects may be in large part related to the fact that they exert most of their actions on the central nervous system, the most complex organ in animals and man. The opiates produce a large variety of pharmacological responses, the most important of which are analgesia, euphoria, and addiction. The latter includes the development of tolerance and physiological and psychological dependence. The discovery of opiate receptors in the central nervous system of animals and man and the resulting discovery of endogenous opiate-like ligands for these receptors (discussed in this issue by L. Terenius and introduced briefly in this review) have given rise to considerable activity and excitement in the field of opiate research.

In this review we attempt to cover, within the limitations of space, the most significant advances in our knowledge of opiate receptors since their discovery.

RECEPTOR POSTULATE AND DISCOVERY OF OPIATE BINDING SITES

The hypothesis that opiates must bind to specific sites located on the surface of or inside nerve cells in order to exert their effects has been put forth by investigators for several decades. These putative binding sites were termed *receptors* in line with the nomenclature used by endocrinologists for specific binding sites for hormones. The concept of a receptor includes, in addition to the binding site, a transducing factor that allows binding to be translated into physical or chemical sequelae that lead ultimately to the observed responses.

The reason for postulating receptors for morphine and related natural and synthetic narcotic analgesics was the high degree of steric and structural specificity

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inherent in many of the actions of the opiates. Thus, for a large number of morphine-like analgesics studied, it is always the levorotatory enantiomer that is active, while the dextrorotatory isomer has little or no analgesic or addiction-producing activity. Small alterations in molecular structure can frequently result in profound changes in pharmacological potency. The most interesting and most studied structural change is the substitution of the N-methyl group by a larger alkyl group such as allyl or cyclopropylmethyl. Such a substitution frequently results in the formation of a molecule with potent, specific antagonistic activity against morphine and related narcotic analgesics. Antagonist activity is most easily explained by competition for a receptor site. Binding of an antagonist is presumed not to produce the subsequent changes necessary to evoke a response. The finding that many such opiate antagonists retain some of their analgesic potency but little of their addiction liability has spurred the synthesis of a large number of such mixed agonist-antagonist drugs for clinical trial as safer analgesics. Antagonists, especially the "pure" antagonists naloxone and naltrexone (devoid of analgesic, addicting, and other opiate "agonist" properties) are also used for the treatment of addicts.

The search for the putative opiate receptors was complicated by the difficulty of distinguishing nonspecific binding to various tissue components from specific binding to receptors. It was relatively easy to demonstrate the binding of tritiated dihydromorphine to brain and other tissue homogenates (1). However, attempts by Van Praag & Simon to distinguish specific binding by its sensitivity to displacement by the antagonist nalorphine were unsuccessful. Ingoglia & Dole (2) were the first to use the principle of stereospecificity in an attempt to identify opiate receptors. They injected *l*– and *d*–methadone into the lateral ventricle of rats, but found no difference in the rate of diffusion of the enantiomers into brain tissue.

In 1971 Goldstein, Lowney & Pal (3) used stereospecificity as the criterion of receptor binding in brain homogenates. They incubated mouse brain homogenates with ^{3}H-levorphanol in the presence of a large excess of unlabeled levorphanol or of its inactive enantiomer dextrorphan. Since dextrorphan has neither agonist nor antagonist activity, it is presumed not to be recognized by the receptor. Stereospecific binding was, therefore, defined as that portion of the binding of the labeled drug which is prevented by levorphanol but not by dextrorphan. In their experiments Goldstein's group found only about 2% of the total binding to be stereospecific. However, in 1973 the laboratories of Simon, Terenius & Snyder (4–6), each using similar modifications of the Goldstein procedure, independently and simultaneously reported the observation of stereospecific opiate binding in rat brain homogenate which represented the major portion of total binding. The modifications involved the use of very low concentrations of labeled ligand, made possible by high specific activity, and the washing of homogenates after incubation with cold buffer to remove contaminating unbound and loosely bound radioactivity. Since that time these results have been confirmed in many laboratories and much evidence has accumulated suggesting that these stereospecific binding sites indeed represent receptors to which opiates must bind in order to produce their pharmacological responses. They have been found in man (7) and in all vertebrates so far studied but not in invertebrates (8).

PROPERTIES OF OPIATE BINDING SITES

The stereospecific binding sites are found in the central nervous system and in the innervation of certain smooth muscle systems such as the myenteric plexus of the guinea pig ileum. They have not been observed in non-nervous tissues. They are tightly associated with membrane fractions of tissue homogenates and have been reported to be most concentrated in the synaptosomal cell fraction (9, 10), suggesting a location in the vicinity of synapses.

Stereospecific opiate binding is saturable, and binding at saturation amounts to about 0.25 pmol per mg of brain protein in whole brain homogenate. Both agonists and antagonists bind with high affinity. Affinities, as defined by dissociation constants, range from 0.4 nM for the potent synthetic analgesic etorphine [more recently certain derivatives of fentanyl have been found to have affinity constants as low as 0.025 nM (11)] to low or no measurable affinity for drugs that possess little or no opiate-like activity. The striking discrimination between stereoisomers is best exemplified by levorphanol and dextrorphan which differ by four orders of magnitude in their affinities (4, 6).

The pH optimum for opiate binding is fairly broad, ranging from 6.5 to 8. The addition of various salts to the incubation medium tends to reduce binding. Sodium represents an exception that is dealt with in some detail in the section on conformational changes in opiate receptors.

The inhibition of stereospecific opiate binding by proteolytic enzymes (4, 12) and a wide variety of protein reagents, including sulfhydryl reagents (4, 13), strongly suggests that one or more proteins are involved in opiate binding. The role of lipids is less clear since binding is inhibited by treatment with phospholipase A (12) but is virtually unaffected by phospholipase C (4, 12). Ribonuclease, deoxyribonuclease, and neuraminidase are without effect on opiate binding (12).

The most convincing evidence indicating that the observed binding sites represent pharmacological receptors comes from the close correlation between binding affinities and pharmacological potencies observed for a large number of drugs in several studies. Thus, good correlation was found for a homologous series of ketobemidones differing only in the length of their alkyl substitution on the nitrogen (14). Creese & Snyder (15) found excellent correlation between binding of a series of opiate agonists and antagonists to receptors in the guinea pig ileum and their pharmacological potency in this system.

Stahl et al (11) studied a series of 26 coded drugs from the repertory of Janssen Pharmaceutica. The binding affinities of these drugs were assessed by competition with labeled naltrexone for stereospecific binding. These affinities were compared to analgesic potencies measured by the tail withdrawal reaction following intravenous administration of drug. In this series, in which the drugs varied in pharmacological potencies over six orders of magnitude, a rank correlation coefficient of 0.9 was found.

The drugs studied included a number of neuroleptics, such as haloperidol and droperidol, which were found able to compete with labeled opiates for binding in the micromolar range, in agreement with reports by others (16, 17). Van Nueten,

Janssen & Fontaine (18) have found that similar concentrations of neuroleptic drugs will inhibit the electrically stimulated contractions of the isolated guinea pig ileum in a manner reversible by naloxone.

Loperamide, an antidiarrheal drug with little effect on the central nervous system, has high affinity for opiate receptors. Experiments were presented (11) suggesting that this drug does not readily penetrate the blood-brain barrier, a property that may explain the separation of antidiarrheal and central effects of this drug.

The stereospecificity, saturability, and high affinity of the opiate binding sites and, above all, the excellent correlation between pharmacological potency and binding affinity for a large number of opiates and antagonists support the hypothesis that these sites represent the binding portion of pharmacologically relevant receptors.

REGIONAL DISTRIBUTION OF OPIATE RECEPTORS

The regional distribution of stereospecific opiate binding in the CNS is of considerable interest because it might reveal a relationship between opiate receptor localization and regional brain function. Hiller, Pearson & Simon (7), therefore, embarked on a detailed study of regional distribution of stereospecific ^{3}H-etorphine binding in human brain obtained at autopsy from the office of the chief medical examiner of the City of New York. Stereospecific binding could be demonstrated in autopsy material, and the level of binding for a given anatomical site from one brain to another showed remarkably good reproducibility. Over 40 anatomical regions were examined for opiate receptor levels. Receptors are not distributed uniformly. As seen in Table 1 there are large differences in levels of etorphine binding ranging from 0.4 pmol/mg protein in, for example, the olfactory trigone, amygdala, and septal nuclei, to virtually no binding in cerebral white matter, dentate nucleus of the cerebellum, tegmentum, etc. Since publication of the above results, eight additional human brains have been examined and a number of new areas have been surveyed (19). High binding levels were found also in the insula, uncus, fusiform gyrus, and the locus coeruleus. Moderate binding was observed in the lingulate gyrus and the paracentral lobule. The anterior portion of the cingulate gyrus had consistently higher binding than the posterior portion. Similarly, binding in the anterior hypothalamus was found to be higher than in the posterior portion.

The most interesting conclusion reached from this study is that many areas that demonstrate high levels of opiate binding are located in, or associated with, the limbic system. This system has frequently been suggested as a possible region of opiate action on the basis of ablation and electrical stimulation experiments (20–22). The only area of the limbic system that does not exhibit high opiate binding is the hippocampus. This finding is in agreement with the report that morphine administration to monkeys elevates the threshold for licking and chewing movements produced by stimulating the amygdala, but no significant changes are seen in the threshold or duration of hippocampal after-discharge (23). Kuhar, Pert & Snyder (24) reported identical results for the regional distribution of dihydromorphine binding in monkey brain. It is of interest that most of the areas of highest concentration of the endogenous opioid peptides, enkephalins, are in the limbic system (25)

Table 1 Grouping of human brain regions according to etorphine binding capacity[a]

High binding (0.44–0.23 pmol/mg protein)	
Olfactory trigone[b]	Centromedian nucleus of thalamus[b]
Amygdala[b]	Preoptic area and supraoptic nucleus[b]
Septal nuclei[b]	Cingulate gyrus[b]
Supraorbital gyrus of frontal lobe[b]	Dorsomedian nucleus of thalamus[b]
Parahyppocampal gyrus[b]	Frontal lobe cortex[b]
Periventricular gray matter[b]	Pulvinar of thalamus
Temporal lobe[b]	
Moderate binding (0.21–0.15 pmol/mg protein)	
Caudate nucleus	Olfactory bulb
Parietal lobe cortex	Periaquaductal gray
Hypothalamus[b]	Putamen
Ventral anterior nucleus of thalamus	Ventral posterolateral nucleus of thalamus
Low binding (0.12–0.07 pmol/mg protein)	
Occipital lobe cortex	Cerebellar cortex
Corpora quadrigemina	Pretectum
Hippocampus[b]	Substantia nigra
Globus pallidus	Area postrema
Very low binding (0.05–0 pmol/mg protein)	
Mammillary bodies	Olives
Medullary sensory nuclei	Dentate nucleus of cerebellum
Cerebral white matter	Tegmentum of mid pons
Posterolateral nucleus of thalamus	Pineal gland
Red nucleus	Pituitary gland

[a] From reference (7), with permission from PJD Publications Ltd.
[b] Indicates components of the limbic system or regions associated with the limbic system.

indicating a correlation between the localization of naturally occurring ligand and receptor.

Binding characteristics of opiate receptors in longitudinal muscle-myenteric plexus preparations from guinea pig intestine are closely similar to the properties of opiate receptors in brain (15). Autoradiographic techniques have shown localization of receptor binding on the satellite cells of the myenteric plexus (26) rather than on the larger ganglion cells.

The demonstration of receptor binding after in vivo administration of labeled opiates proved to be difficult. However, such binding has now been observed in several laboratories and has been defined as binding of labeled opiates that can be released by the administration of opiate antagonists. Mulé, Casella & Clouet (27) were able to show partial displacement of ^{3}H-etorphine from a synaptic membrane fraction of rat brain by naloxone, after either peripheral or central administration of etorphine. Similar results have been obtained by Cerletti, Manara, & Mennini (28) who found reductions in brain levels of ^{3}H-etorphine, administered intravenously,

when animals had been pretreated with the antagonists cyprenorphine, nalorphine, or naloxone. Reduction was seen in cerebral hemispheres, brain stem, and spinal cord, but not in the cerebellum. No reduction in etorphine level was seen after pretreatment with agonists such as heroin, morphine, or unlabeled etorphine. Höllt et al (29) have demonstrated that the brain level of ^{3}H-naltrexone is reduced up to 60% by the simultaneous injection of unlabeled naltrexone or levallorphan. Dextrallorphan, the inactive enantiomer of levallorphan, was ineffective, confirming the stereospecificity of the displacement. Shen & Way (30) have reported a dramatic decrease in brain levels of morphine when morphine-addicted mice were treated with naloxone. This effect was not seen in naive mice.

Pert & Snyder (31) used another technique for the measurement of opiate binding after in vivo administration. They showed that rapid homogenization of brain tissue followed by immediate filtration and washing of filters allowed discrimination between specific and nonspecific binding. Nonspecific binding was defined as the amount of radioactivity retained on the filters when homogenate of cerebellum, a brain region previously shown to be essentially devoid of opiate receptors, was filtered. Specific binding had the characteristics of receptor binding observed in vitro, namely saturability, high affinity, specific displacement by unlabeled opiates or antagonists, similar number of binding sites, and comparable regional distribution.

The distribution of stereospecific binding levels after in vivo administration of labeled opiates has also been examined by autoradiography. The very potent antagonist ^{3}H-diprenorphine was utilized by Pert, Kuhar & Snyder (32). Stereospecificity of the system was shown by the ability of levallorphan, but not dextrallorphan, to reduce total brain content of ^{3}H-diprenorphine by 75%. Translocation of the ligand by diffusion away from the receptor site was greatly minimized by fixation of tissue slices at low temperature. The regional distribution of the opiate receptor in the brain as determined by in vitro methods was confirmed by the autoradiographic results. In addition, dense silver grain areas were found in a "streak" ventral to the corpus callosum, in the zona compacta of the substantia nigra, in the locus coeruleus, and in the substantia gelatinosa of the spinal cord.

The high grain count in the locus coeruleus confirms our earlier report (19) that a high level of binding was present in the locus coeruleus of human brain and adds interest to the report of Korf, Bunney & Aghajanian (33) that small doses of morphine slowed the firing rate of locus coeruleus cells but not that of adjacent cells. Similar results from autoradiographic investigations were obtained by Shubert, Höllt & Herz (34) who injected the potent agonist ^{3}H-etorphine into mice. More detailed autoradiographic studies by Atweh & Kuhar (35) demonstrated that opiate receptors are highly localized in the dorsal horn of the spinal cord in layers I (marginal cell zone) and II (substantia gelatinosa) and the spinal trigeminal nucleus which is consistent with other evidence for analgesic effects of opiates at the spinal level. In the lower medulla, heavy concentrations of opiate receptors were found in the visceral afferents of the vagus and glossopharyngeal nerves which may be the possible site of the antitussive effects of opiates. Also in the lower medulla, high binding has been found in the area postrema, a possible region of opiate-induced nausea and vomiting. In the brain stem (36) dense localization of receptors has been

seen in areas that are related to the accessory optic pathway which includes the superior colliculus, the pretectal nuclei, and the ventral lateral geniculate body. In the thalamus, the infundibulum, medial thalamus, and habenulo-interpeduncular complex showed dense concentrations of opiate receptors. Areas in the telencephalon showing high levels of opiate receptor included parts of the presubiculum and amygdala, patchy areas in the caudate-putamen and accumbens, the subfornical organ, the interstriatal nucleus of the strial terminalis, and the anterior olfactory nucleus pars externa (37).

Early effects in mammals after acute morphine administration include changes in behavior. While depressive behavior is observable in many species including dog, monkey, and man, excitatory behavior is elicited in cat, sheep, and cow among others (38). In an effort to ascertain whether the species difference in behavior caused by morphine is reflected in the distribution pattern of opiate binding sites in the brain, Hiller & Simon (39, 40) undertook a survey of the levels of stereospecific binding of ^{3}H-etorphine in selected areas of the brains of the above species. There was reasonably good reproducibility of binding levels for any given anatomical region of all six species studied. The only areas that exhibited consistent differences were the amygdala and the frontal cortex, which were at least twofold higher in receptor level for the species that exhibit depression than for the species that show an excitatory response to opiates.

These consistent differences between the two groups of animals, though based on a small number of species, are intriguing; however, their interpretation is difficult. Both the amygdala and frontal cortex are part of the limbic system wherein most of the regions of high opiate binding in man and monkey are located. It has been reported that bilaterally amygdalectomized monkeys were rendered placid (41), while the same procedure performed on cats produced a sustained ferocity (42). Thus, amygdalectomy mimics the effects seen in acute morphine administration to these species. The removal of the frontal cortex in man may leave the perception of pain unaffected while the anxiety associated with pain is markedly diminished (43). This observation has also been made in patients in pain who are receiving morphine.

ONTOGENY OF THE OPIATE RECEPTOR

The ontogenetic development of opiate receptors in the brain of rat and guinea pig was investigated by Clendeninn, Petraitis & Simon (44). In the rat, the rate of increase of opiate binding is greatest between the midfetal stage and three weeks postpartum (three- to fourfold). Thereafter, until adulthood (10-20 weeks) the rise is more gradual (about twofold). Scatchard analysis of saturation curves for naltrexone binding in rat brain from one day after birth to the age of ten weeks has shown that the increase in binding is due to an increase in number of receptors rather than to enhanced affinity.

Opiate binding to guinea pig brain homogenates has demonstrated no significant difference in either receptor number or binding affinity between late fetal life and adulthood in this species. Binding in brain homogenate from a midterm fetus is about one half that observed late in pregnancy and in adult guinea pigs. The fact

that guinea pigs are born with a full complement of receptors is well correlated with previous reports that the guinea pig is an animal with almost full brain development before birth whereas the rat is an animal in which a significant portion of brain development continues for at least three weeks after birth. In rat brain, the percentage increase of opiate receptors from newborn to adults in various regions of the brain differs widely (45). For example, receptor binding in the hippocampus and parietal cortex increases by 830% and 690%, respectively, but in the medulla pons and corpus striatum by only 270% and 300%.

The development of the opiate receptor in both rat and guinea pig closely parallels the development of other major neurological and biochemical components of the nervous system, suggesting that these receptors are themselves important components of the CNS.

DISCOVERY OF ENDOGENOUS OPIATE-LIKE PEPTIDES

The pharmacology of endogenous opioid peptides is discussed in detail in this volume by L. Terenius. However, a review of opiate receptors would be incomplete without a brief description of the isolation of endogenous ligands for the opiate receptor. Moreover, in several sections of this review we make reference to these peptides.

The discovery that opiate receptors exist in the CNS of all vertebrates from the hagfish to man raised the question why receptors for alkaloids, present only in plants, should exist in the nervous system and have survived the eons of evolution. A physiological role seemed to be the most reasonable postulate and the search for endogenous ligands for the opiate receptor began. When a survey of known neurotransmitters, neuromodulators, and related substances proved negative, the possibility was considered that the natural ligand was an as yet unidentified opioid material present in animal brain. This idea received support from experiments by Liebeskind and his collaborators. These workers had obtained evidence for the existence of a central pain-suppressive system by demonstrating the production of analgesia by electrical stimulation of certain brain areas, such as the mesencephalic central gray and periventricular gray regions (46, 47). This analgesia was shown to be reversed by the specific opiate antagonist naloxone (48). Furthermore, electrically induced analgesia exhibits cross-tolerance with morphine-induced analgesia (49). These results are most readily explained by the existence of an endogenous opioid substance that can be released by electrical stimulation.

The discovery of an endogenous factor in extracts of pig brain which had opiate-like properties in the in vitro bioassay systems (the mouse vas deferens and the guinea pig ileum) was first reported by John Hughes (50, 51), working in the laboratory of Hans Kosterlitz. Terenius & Wahlström (52, 53) independently isolated a water-soluble material from rat and calf brain which was characterized by its ability to compete with labeled opiates for receptor binding. The two materials appeared to be very similar, as was an opioid material extracted somewhat later from calf brain by Pasternak, Goodman & Snyder (54). All of these endogenous opioid substances were water-soluble, heat-stable, degradable by peptidases, and had a

molecular weight of 800–1200. A larger opioid factor was isolated by Goldstein and his collaborators (55, 56) from bovine pituitary glands as well as from crude preparations of ACTH. It was sensitive to trypsin and chymotrypsin and seemed to be a longer polypeptide.

The active factor present in extracts of pig brain has been purified and characterized by Hughes et al (57) and consists of the two pentapeptides H-Tyr-Gly-Gly-Phe-Met-OH and H-Tyr-Gly-Gly-Phe-Leu-OH, named by the Aberdeen group methionine (Met) and leucine (Leu) enkephalin, respectively.

The amino acid sequence of Met-enkephalin was found to be identical with residues 61–65 of the pituitary hormone β-lipotropin (βLPH) (57), isolated from pituitary glands by C. H. Li in 1965 (58).

This remarkable finding gave rise to the isolation of a number of other peptides with opioid properties from extracts of hypothalami and pituitary glands. When the amino acid sequences were determined, all were found to be present in βLPH. The proliferation in the number of endogenous opiate-like peptides caused one of the authors of this review (EJS) to suggest the generic term *endorphin* (for endogenous morphine-like material), which has been widely accepted. Guillemin's group (59, 60) isolated two peptides from pig hypothalamic-neurohypophyseal extracts, α-endorphin, which is sequence 61–76, and γ-endorphin, which represents sequence 61–77 of βLPH. Cox, Goldstein & Li (61) and Bradbury et al (62) independently found that the C-terminal fragment of βLPH (βLPH 61–91) was the most potent endogenous opioid peptide so far isolated. It was renamed β-endorphin by C. H. Li.

All of the endorphins, including the enkephalins, behave like opiate agonists in the binding assay (binding is decreased substantially by the presence of sodium salts) and in the bioassays. They exhibit a variety of opiate-like effects when injected into various brain regions (analgesia, catatonia, hypothermia, respiratory depression, etc).

It should be noted that in spite of the enormous research activity in this field no proof is yet available that any of the endorphins have a physiological function. There is, however, a general consensus that such functions will be found. The suggestion has been made that the rapidly degraded enkephalins may be novel neurotransmitters involved in a natural pain-suppression system or possibly also in other behavioral and emotional functions. The more stable pituitary endorphin polypeptides of greater length are thought to be good candidates for a role as neuromodulators, although a role solely as precursors for enkephalins has not been ruled out.

CONFORMATIONAL CHANGES IN OPIATE RECEPTORS

The studies discussed so far do not permit distinction of a potent agonist from an equally potent antagonist by a receptor binding assay. Both types of drugs compete with each other for the same receptor and show identical binding characteristics. A way that permits the distinction of agonists from antagonists by their binding characteristics came from what appeared to be an experimental discrepancy between two laboratories. Pert & Snyder (6) reported that the addition of salt had little effect

on binding, while Simon, Hiller & Edelman (4) reported profound inhibition. Since the NYU group was using the potent agonist etorphine and the Hopkins group was using the "pure" antagonist naloxone, Simon, Hiller & Edelman (4) suggested that the apparent discrepancy might represent a general difference in the manner in which agonists and antagonists bind to the receptor. It was indeed found that the binding of all agonists examined was inhibited by salt while the binding of antagonists was enhanced. It was also shown (63) that this ability to "discriminate" between agonists and antagonists was not a general effect of salts, but a very unique property of the cation Na^+. The effect is not shown by the other alkali metals K^+, Rb^+, and Cs^+, while Li^+ does exhibit this action but to a much smaller degree. No other inorganic cations nor a series of organic cations studied (64) were found to exhibit this property. This ability of sodium ions to "distinguish" between such closely related molecules as an opiate and its corresponding antagonist (allyl or cyclopropylmethyl analogue) is of considerable interest, especially in light of the uniqueness of Na^+ in this respect. The effect of sodium reaches its maximum at 100 mM and is completely reversible upon removal of sodium from the incubation medium.

Manganese and magnesium salts have been reported to enhance agonist binding while they depress antagonist binding (65). This effect is observed most clearly when sodium is also present and may represent a reversal of the sodium effect by the divalent cations.

Studies of the mechanism of the sodium effect were carried out. The first question asked was whether the differences in binding represented changes in the number of sites or in the affinity of binding. Pert & Snyder (63) reported that sodium caused an increase in the number of high affinity binding sites for naloxone and a decrease of binding sites for dihydromorphine. Simon et al (64), on the other hand, found that sodium increased the affinity for the receptor of naltrexone and other antagonists while reducing the affinity of agonists. No change in the number of binding sites was noted. These results were consistent with a model involving a conformational change of the opiate receptor in the presence of sodium ions. The experimental discrepancies have never been completely resolved. However, studies carried out in both laboratories (63, 64) involving competition experiments favor the changes in affinity. When a relatively pure antagonist is allowed to compete for binding with a labeled antagonist there is little or no change in the IC_{50} of the competitor when sodium is added to the incubation mixture. When an unlabeled agonist is allowed to compete with a labeled antagonist the IC_{50} for the agonist is increased drastically in the presence of sodium (10- to 60-fold). Such a shift in IC_{50} reflects a change in affinity but not in the number of binding sites.

The best evidence for an alteration in receptor conformation by sodium ions came unexpectedly from a study of the kinetics of receptor inactivation by the sulfhydryl alkylating reagent, N-ethylmaleimide (NEM) by Simon & Groth (66). When a membrane fraction from rat brain was incubated with NEM for various periods, followed by inactivation or removal of unreacted NEM, there was a progressive decrease in the ability of the membranes to bind opiates stereospecifically. The rate of receptor inactivation followed pseudo-first order kinetics consistent with the

existence of one SH-group per receptor essential for binding. Protection against inactivation was achieved by the addition of low concentrations of opiates or antagonists during the preincubation with NEM, suggesting that the SH-group is located near the opiate binding site of the receptor.

Considerable protection was observed (half time of inactivation was increased to 30 min from 8 min) when inactivation was carried out in the presence of 100 mM NaCl. Since sodium salts were without effect on the alkylation of model SH-compounds, such as cysteine or glutathione, this suggested that the SH-groups were made less accessible to NEM by a conformational change in the receptor protein. The fact that this protection exhibited the same ion specificity (Na^+ protects, Li^+ protects partially, K^+, Rb^+, or Cs^+ not at all) and the same dose-response to Na^+ as the differential changes in ligand affinities, suggests that the conformational change that masks SH-groups is the same as that which results in increased affinity of antagonists and decreased affinity of agonists.

These studies illustrate that the opiate receptor can alter its shape. The physiological function of this plasticity is not yet clear. A role in the coupling of opiate binding to subsequent physical or chemical events has been suggested as has a role for Na^+ ions in the action of opiates.

It should be mentioned that similar changes have been observed as a consequence of changes in the temperature at which binding takes place. This was first shown by Creese et al (67) and confirmed by Hiller & Simon (39).

Evidence for positive cooperativity has been obtained at least for the binding of antagonists in the presence of NaCl (68). In this study a Hill coefficient of 1.5–2 was observed. More recently Davis et al (69) reported a very high degree of cooperativity for the binding of both agonists and antagonists (Hill coefficient > 3). This, however, was observed only when binding was carried out with brain slices and not when homogenized (broken cell) preparations were used.

A simple model for the allosteric effect of sodium on opiate receptors (19) is shown in Figure 1. In line with the observed cooperativity the receptor is represented as a dimer. When sodium ions are bound to the allosteric site there is a change in the shape of the receptor molecule. This, in turn, results in an alteration in the binding site which now binds antagonists with greater and agonists with reduced

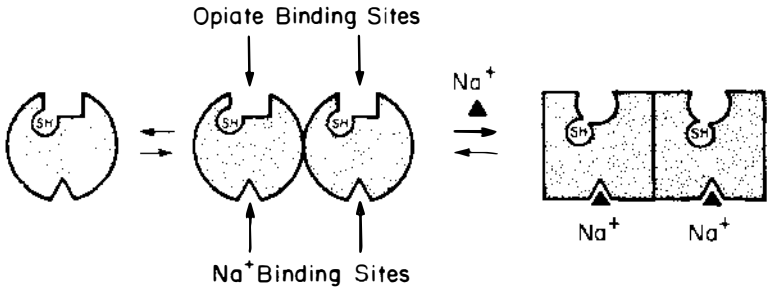

Figure 1 Model for the allosteric effect of sodium ions on the conformation of the opiate receptor.

affinity. It also results in a "masking" of the SH-group of the receptor that makes it less accessible to inactivation by SH-reagents.

The dissociation of the dimeric receptor into its subunits is presented as one possible explanation for the observed persistence of some "Na^+-free" conformer in the presence of 100 mM NaCl (64). If the monomer is unable to bind Na^+ ions or to undergo the resultant conformational change, and if redimerization is very slow, this model could account for the incomplete conversion to the "Na^+-dependent" conformational state. Other explanations of this phenomenon are possible.

PROGRESS IN SOLUBILIZATION AND PURIFICATION OF OPIATE RECEPTORS

The isolation and purification of the membrane-bound opiate receptors has proved to be difficult. The fact that these receptors appear to be unusually sensitive to detergents, even the non-ionic variety, has complicated attempts at solubilization. The presence of only very small concentrations of opiate receptors in even the richest brain regions and the absence of an effective, specific affinity labeling material to date have further contributed to the relatively modest progress in this important endeavor.

H. K. Lin and E. J. Simon (unpublished results) have shown that sonication can provide a good yield of small membrane fragments that no longer sediment at 65,000 X g, a small portion of which do not sediment even at 100,000 X g. However, the molecular weights of these nonsedimentable receptors are still in the many millions, suggesting that they are very small membrane fragments of low density (high lipid content).

A number of opiate affinity chromatography columns have been prepared in Simon's laboratory (70, 71). While their use for purification of antimorphine antibodies has been quite successful (72), they have not yet yielded enrichment of receptor-carrying membrane fragments.

The most promising advance has been the solubilization by Simon, Hiller & Edelman (73) of an etorphine-bound macromolecule which has properties consistent with those of an etorphine-receptor complex. This solubilization was achieved by the use of the non-ionic detergent Brij 36T® and the use of columns of Amberlite XAD-2® for the separation of free and bound ^{3}H-etorphine. As much as 25–30% of prebound (in presence of dextrorphan) radioactivity present in the supernatant from ultracentrifugation at 100,000 X g appeared in the void volume of the XAD column as bound etorphine. When binding was carried out in the presence of excess unlabeled levorphanol or etorphine, virtually no bound radioactivity appeared in the solubilized protein fraction, providing evidence for the stereospecificity of the solubilized macromolecular complex. The bound etorphine was released by proteolytic enzymes, heat, and sulfhydryl reagents suggesting the participation of protein in binding. The molecular weight of the solubilized complex determined on a calibrated Sepharose 6B column was about 400,000. All the evidence so far obtained is consistent with the notion that this solubilized material is an etorphine-receptor complex.

Numerous attempts to obtain an active solubilized receptor, i.e. one that can bind opiates after its removal from the membrane, have so far met with very limited success. Some stereospecific binding is observed when solubilization is carried out in a similar manner but without prebinding of labeled etorphine. However, the amount of binding observed is small, and reproducibility from experiment to experiment is poor (H. K. Lin, J. M. Hiller, and E. J. Simon, unpublished results).

OTHER TYPES OF BINDING MATERIAL FOR OPIATES

Goldstein and his collaborators (74) followed up their observation of low levels of stereospecific binding by demonstrating that a material that binds opiates stereospecifically was extractable into chlorofor-Methanol. They proceeded to purify this substance which appeared to have the properties of a proteolipid. It differed markedly from the receptors studied in the laboratories of Terenius, Snyder, and Simon. Thus, the Goldstein material binds levorphanol with an affinity two orders of magnitude lower, it is insensitive to proteolytic enzymes and sulfhydryl reagents, and the binding sites have a quite different distribution. High levels were found in the cerebellum and the brain stem, where the level of the high affinity receptors is low.

The interesting observation was made by Loh et al (75) that cerebroside sulfate obtained from commercial sources was able to bind labeled opiates and antagonists stereospecifically with affinities comparable to those reported by Goldstein's group for their proteolipid. Convincing evidence has been obtained by Loh's team (76) that the material isolated in Goldstein's laboratory was, in fact, largely cerebroside sulfate.

The binding of opiates to cerebroside sulfate has been studied intensively by Loh et al (76). Good correlation has been found between binding affinities and analgesic potencies. Correlation was particularly good for a homologous series of N-alkylnorketobemidones. Loh and co-workers regard cerebroside sulfate as a valuable in vitro model for the study of stereospecific opiate binding. They are also actively continuing their efforts to determine whether cerebroside sulfate may be involved in opiate binding in vivo. The finding that a strain of mice called *Jimpy,* deficient in brain cerebrosides, requires higher levels of morphine for analgesia than normal mice lends some support to this idea (76).

Very recently Loh et al (77) reported the use of ^{125}I-3-iododiazosulfanilic acid (^{125}I-DISA) as an affinity label for the opiate receptor. Membranes were treated with unlabeled DISA while the receptors were protected with dextrorphan plus or minus morphine. After removal of the opiates the membranes were treated with ^{125}I-DISA. Total lipids extracted with chloroform-methanol were separated by thin-layer chromatography (TLC) in four solvent systems. The authors report that in all solvent systems used the membranes protected by dextrorphan plus morphine showed a significant increase in radioactivity at an R_f corresponding to that of ^{125}I-DISA-labeled cerebroside sulfate, when compared to membranes protected with dextrorphan alone. Since diazosulfanilic acid inhibits stereospecific binding of labeled opiates in a concentration-dependent manner, these findings provide further

support for the idea that cerebroside sulfate may be involved in opiate receptor binding.

Abood & Hoss (78) have reported the binding of opiates to phosphatidyl serine (PS). Binding to other acidic phospholipids exhibits much lower affinity. The addition of PS to membrane preparations from rat brain was found to stimulate stereospecific binding of opiates by about 35% (79). It is suggested that this represents a direct effect on the receptor since similar results were obtained with the ethyl glycolate ester of PS which, unlike the free phospholipid, is unable to bind opiates or Ca^{2+} (80).

BIOCHEMICAL EVENTS FOLLOWING OPIATE BINDING

As indicated earlier, the binding of opiates to their receptors must trigger chemical or physical events that ultimately result in the observed pharmacological responses. The nature of these events remains a "black box." However, efforts have been made to penetrate the box and are summarized below.

Cyclic 3', 5'-adenosine monophosphate (cAMP) has been shown to act as "second messenger" in a number of hormonally controlled phenomena. The finding that cAMP and the phosphodiesterase inhibitor, theophylline, antagonize the antinociceptive action of morphine (81) suggested to Collier & Roy that cAMP may have a role in the action of opiates. These authors have shown (82) that the stimulation of cAMP formation by prostaglandins E_1 or E_2 in rat brain homogenate is inhibited by morphine and other opiates. There is no inhibition of the basal production of cAMP. This inhibition was seen at concentrations of opiates comparable to those required for analgesia and was antagonized by naloxone. The study of a series of opiates (83) led to the finding that inhibition of prostaglandin-stimulated formation of cAMP was well correlated with antinociceptive potency, opiate receptor binding affinity, and inhibition of electrically stimulated contraction of the isolated guinea pig ileum. Specificity for E-prostaglandin stimulation was suggested by the absence of inhibition of fluoride-stimulated cAMP production. Involvement of E prostaglandins is further supported by the finding of Ehrenpreis, Greenberg & Belman (84) that they reverse inhibition by morphine of contractions of the guinea pig ileum.

Similar results were obtained in the neuroblastoma X glioma hybrid cells in culture (85, 86). Here, however, there appears to be less specificity since Sharma et al (85) have found that opiates inhibit basal as well as adenosine and PGE_1-stimulated adenylate cyclase (see section on cell and tissue culture).

In apparent contradiction to the results of Collier's group, Puri, Cochin & Volicer (87) have reported that morphine sulfate produced a dose-dependent increase in the adenylate cyclase of rat corpus striatum when given in vivo or added to the enzyme in a striatal homogenate. However, this effect is not stereospecific and cannot be reversed by naloxone (88). Iwatsubo & Clouet (89) reported that the addition of morphine and other opiates (3–300 mM) to crude synaptosomal membranes from rat caudate nucleus had no effect on the dopamine stimulation of adenylate cyclase. However, when morphine was administered subcutaneously to rats at a dose of 60

mg/kg, significant increases were found in basal and dopamine-stimulated adenylate cyclase in the caudate nucleus from 15 to 120 min after injection.

Bonnet (90) has reported that systemic injections of morphine produced dose-dependent increases in the level of cAMP and adenylate cyclase in the striatum and thalamus of rat brain, whereas in the substantia nigra and the periventricular gray area the level of cAMP was found to be reduced. More recently Bonnet & Gusik (91) have demonstrated that the increases in thalamic adenylate cyclase were reversible by naloxone, suggesting the involvement of opiate receptors in this effect. They also found that the level of Ca^{2+} was of great importance. Physiological levels of Ca^{2+} (up to 1.3 mM) tended to enhance the opiate stimulation of adenylate cyclase in the thalamus, while high levels of Ca^{2+} tended to suppress it.

Minneman (92) reported in a very recent communication that morphine, at concentrations from 10^{-7} to 10^{-4} M, caused a 30–50% decrease in cAMP levels of striatal slices. He also found a highly specific and complete inhibition of dopamine-stimulated cAMP levels in the slices. Morphine, up to a concentration of 10^{-3} M, had no effect on the stimulation of cAMP levels elicited by isoprenaline, adenosine, or PGE_1. This selective inhibition of dopamine-sensitive adenylate cyclase, as well as the depression of basal cAMP, was not seen in striatal homogenate and appears, therefore, to require intact cells. Both effects were blocked by naloxone (1 μM) suggesting that they are mediated via specific opiate receptors.

The finding by Ho, Loh & Way (93) that the administration of cAMP accelerated the development of tolerance and physical dependence stimulated exploration as to whether raised levels of cAMP could produce behavioral patterns in naive rats resembling the morphine-withdrawal syndrome. Collier and collaborators approached this by administration of large doses of phosphodiesterase inhibitors such as theophylline and other methyl-xanthines. Such behavior, termed by the authors *quasimorphine-withdrawal-syndrome* (QMWS), was indeed observed (94), as was intensification of the syndrome by opiate antagonists and its stereospecific suppression by agonists (95). It has not been proven that the observed effects are indeed the result of raised brain levels of cAMP. However, the testing of seven phosphodiesterase inhibitors showed that ability to elicit the QMWS was correlated with their potency as inhibitors of cAMP hydrolysis (96). Moreover, there is a report by Mehta & Johnson (97) that total brain content of cAMP increases sharply during naloxone-precipitated withdrawal from opiates. The effect of long-term morphine treatment and withdrawal on cAMP production in neuroblastoma × glioma hybrid cells in culture is discussed below in the section on cell and tissue culture.

Assuming that opiates (and endorphins) can indeed produce changes in the cellular level of cAMP and assuming that some of the contradictory results are sorted out and reconciled, the manner in which these changes are implicated in either acute or chronic effects of opiates is still unclear. However, as yet unpublished results from several laboratories (K. A. Bonnet, D. H. Clouet, P. Greengard personal communications) suggest that the cAMP-dependent phosphorylation of membrane proteins may be affected by chronic morphine treatment of animals.

The results suggesting that the opiate receptor can exist in alternate conformations make it attractive to postulate that similar physical chemical changes may

occur which could mediate opiate effects by causing modifications in the neighboring synaptic membrane. To date, the evidence for such alterations during acute or chronic treatment with opiates is scant. Kang, Sessa & Green (98) reported that synaptosomal membranes, isolated from rat brain cortex, undergo a characteristic structural change accompanied by a time-dependent increase in ultraviolet absorption (at 265 and 220–230 nm) and a decrease in intrinsic fluorescence intensity. Morphine sulfate appears to block this transition as shown by inhibition of the optical density increase at 265 nm. This effect, however, required relatively high concentrations of morphine (95 μg/ml) and was not tested for stereospecificity. Preliminary results obtained in our laboratory indicate that quenching of fluorescence in partly purified synaptosomal membranes can be produced by the addition of levorphanol but not of dextrorphan (N. Clendeninn and E. J. Simon, unpublished results).

STUDIES IN CELL AND TISSUE CULTURE

Cell cultures with their relatively homogeneous cell population have long been investigated as possible useful model systems for the elucidation of underlying mechanisms of opiate action and of development of tolerance and dependence. The earlier work in this area has been reviewed (99).

Opiate receptors have been demonstrated in a neuroblastoma X glioma hybrid cell line (NG108-15) by Klee & Nirenberg (100). The parental lines, in contrast, showed little or no binding. The opiate receptors of this hybrid cell line had binding affinities for a variety of opiate ligands that correlated well with the pharmacological potency of the various compounds, and were approximately the same as (or somewhat lower than) those seen in rat brain homogenates. More recently a sympathetic ganglion X neuroblastoma hybrid cell line has been reported to possess high levels of opiate receptors (101).

The reports of Collier & Roy (82, 83) that prostaglandin E_1 (PGE_1) sensitive adenylate cyclase was inhibited by morphine in rat brain homogenates led other investigators to test this phenomenon in the neuroblastoma X glioma hybrid and its parental lines (85, 86). It was indeed found that morphine and other opiate agonists inhibit the stimulation by PGE_1 and by adenosine of the formation of cAMP in the hybrid cell line. Furthermore, affinity of opiate agonists for the receptor correlates well with their efficacy in inhibiting adenylate cyclase. This inhibition proved to be noncompetitive; that is, high concentration of PGE_1 did not overcome the inhibitory action of morphine, indicating that PGE and morphine have separate receptor sites (86). However, kinetic studies demonstrated that adenylate cyclase and morphine interact cooperatively as evidenced by a Hill coefficient of 2.3 (85), although, in contrast to findings in brain homogenate (68), cooperativity was not observed for receptor binding. The ability of opiate agonists to inhibit PGE_1-stimulated adenylate cyclase is seen to a small degree in the neuroblastoma parental line that contains a small number of receptors, but not at all in the glioma parental line which is totally devoid of opiate receptors. Therefore, the ability of opiate agonists to inhibit this enzyme appears to vary with the number of opiate receptors present in a cell line.

In contrast to the findings in rat brain homogenate (82), it was demonstrated that morphine also reduces basal levels of cAMP in intact hybrid cells and inhibits basal adenylate cyclase activity in cell homogenates (85, 86). These effects were shown to be stereospecific and antagonized by naloxone, which by itself had little effect on either basal or PGE_1-stimulated adenylate cyclase. It has recently been shown (102, 103) that enkephalins can also lower the basal and PGE_1-stimulated levels of adenylate cyclase in the hybrid cell line.

Effects of chronic morphine treatment has also been observed in these cells. When the hybrid cells were cultured in the presence of morphine for four days, there was a gradual rise in both basal and PGE_1-stimulated adenylate cyclase (104). After two to three days both basal and PGE_1-stimulated adenylate cyclase measured in the presence of morphine were about the same as in control cells, preincubated and assayed in the absence of morphine. Sharma, Klee & Nirenberg (104) suggested that this may be the cells' equivalent to tolerance in animals and man. Adenylate cyclase was abnormally high in morphine-pretreated cells when assayed in the absence of opiates. This was demonstrated even more dramatically when cAMP levels were measured in cells exposed briefly to the antagonist naloxone after pretreatment with morphine for several days. Such cells showed as much as a fivefold increase in cAMP levels, a phenomenon suggested as analogous to precipitated withdrawal in animals and man. Adenylate cyclase levels were shown to return to normal within 24 hr after withdrawal of morphine from the culture medium. This cell line had also been shown to become tolerant to and dependent on the endogenously occurring opiate peptide, methionine enkephalin (105).

Traber, Gullis & Hamprecht (106) have made the similar observation that preincubation of cells for several hours with morphine strongly enhances their response to PGE_1, as expressed by an increased cellular level of cAMP. Such an enhancement also results from preincubation with methadone and the transmitter analogues isoproterenol and carbamylcholine. Dextrorphan and naloxone had no long-term effect, nor did, curiously enough, levorphanol. The authors suggest that the increased concentration of opiate antagonist required to compensate for the enhancement by PGE_1 of cAMP levels in cells preincubated with opiates is the equivalent of tolerance, while the increased level of cAMP observed when pretreated cells are challenged with PGE_1 in the absence of morphine is thought to be equivalent to the abstinence syndrome in animals and man.

It will be of great interest to learn to what extent these findings in cell culture apply to the action of opiates in the central nervous system. Meanwhile, however, the NG108-15 hybrid cell line presents investigators with a new biological assay system for the testing of both synthetic and naturally occurring compounds for opioid activity by their ability to inhibit PGE_1-stimulated adenylate cyclase in a naloxone reversible manner.

For the investigation of problems related to the mode of action of opiates in the CNS, organotypic cultures of central nervous tissue would clearly provide advantages over tumor-derived established cell lines. Such cultures derived from fetal mouse spinal cords and attached sensory ganglia (107) have recently been found useful for studies of opiate action. Crain et al (108) demonstrated that the sensory-

evoked synaptic networks in the dorsal horn regions of these explants can be selectively depressed by exposure to analgesic concentrations of morphine and other opiates. These effects are reversed by naloxone.

Measurements of opiate receptor binding levels in these spinal cord–dorsal root ganglion cultures as well as in cultures of isolated dorsal root ganglion and deafferented cord explants were made by Simon et al (109). In these cultures, profuse neuritic outgrowth develops, primarily as a result of the stimulation by nerve growth factor of the dorsal root ganglia, and extends for several millimeters beyond the explant zone. In some cases separate determinations of opiate binding were made on explant and outgrowth zones. Homogenates of these cultures were found to exhibit stereospecific binding of the potent opiate antagonist diprenorphine. It was apparent that the greatest amount of binding was present in the neuritic outgrowth of both isolated dorsal root ganglion cultures and cord-ganglion cultures. Four to seven times more binding per milligram of protein was seen in the neuritic outgrowth of the cultures than in the explant area.

The central afferent branches of the neuritic arborization from the dorsal root ganglion explant have been shown in cord-ganglion cultures to establish synaptic connections within the dorsal horn of the spinal cord explant. The high level of opiate receptors in the neuritic outgrowth constitutes strong evidence for a presynaptic location of these receptors.

Experiments in rhesus monkeys demonstrated a reduction in opiate receptor binding in the upper dorsal horn of the spinal cord following dorsal root section (110). This result also supports a presynaptic location on primary afferent terminals in the spinal cord. However, as pointed out by the authors, a postsynaptic localization of receptors cannot be ruled out since the rhizotomy-induced decrease in opiate binding could result from transsynaptic degenerative changes.

ENVIRONMENTAL EFFECTS ON OPIATE RECEPTORS

Tolerance occurs to the analgesic and behavioral effects of opiate analgesics after repeated encounter, with a concomitant increase in sensitivity to opiate antagonists. The hypothesis (111) that tolerance is due to a change in the number or affinity of the receptors has been tested in whole brain homogenates, but no change in the number or affinity of binding sites has been demonstrable (9, 112). However, if such changes were to account for tolerance, they could be masked in experiments in which whole brain was used rather than discrete brain regions. Bonnet, Hiller & Simon (113), therefore, attempted to measure changes in the number and relative affinity of receptors for agonist and antagonist binding in the caudate nucleus, medial thalamus, and periaquaductal gray region (areas suggested to be involved in the tolerance and dependence phenomena) of control and tolerant/dependent rats. No alterations in either the number of binding sites or in binding affinity could be detected in these areas.

Davis, Akera & Brody (114) have recently published results indicating that chronic morphine treatment decreases the affinity of the opiate receptor for morphine. These results were obtained in rat brain stem slices but were not observed

in homogenates. Differences in affinity of antagonists have not yet been measured in such slices.

A behavioral, nonpharmacological treatment consisting of rearing mice from weaning in social isolation for five months has been shown to alter the opiate receptor population (112). Control mice were reared in conditions of aggregation. A 20% reduction of agonist binding was seen in brain homogenates of "isolated" mice. Scatchard analysis of ^{3}H-etorphine saturation curves suggested that the number of binding sites in brains of isolated mice was reduced relative to sites in brain of aggregated mice while the affinity remained unchanged. Parallel pharmacological studies indicated that isolation-reared mice demonstrated a decreased sensitivity to the analgesic effects of morphine when tested by the hot plate procedure. Another report (115) has shown that differential housing can alter the naloxone-precipitated abstinence syndrome in morphine-dependent rats previously subjected to long-term isolation.

CONCLUDING COMMENTS

Research on the mode of action of opiates received an enormous boost by the discovery of the long postulated opiate receptors and by the consequent discovery of endogenous opiate peptides, endorphins, which are natural ligands for opiate receptors. Although the field is moving at a rapid pace there is much yet to be learned.

Is there a single type of receptor with different functions in different brain areas or are there multiple receptors, each of which mediates one or more of the many responses to opiates? What is the physiological role of the sodium effect and of the ability of opiate receptors to exist in alternate conformational states? What is the nature and sequence of events that occur between the binding of opiates to the receptor and the pharmacological response? What is the chemical nature of the receptor? The answer to the last question must await the purification of an active receptor or of an affinity-labeled receptor. The solubilization of an etorphine-receptor complex would appear to be a significant step in this direction.

The discovery of the endogenous opioid peptides has added a new dimension to investigations of the opiate receptor by increasing the likelihood that this receptor is an important functional constituent of the CNS of all vertebrates.

As has been stated earlier, the physiological functions of the various endorphins are still unknown. In fact, there is as yet no evidence for a physiological role, even though the probability of such a function for one or more of these peptides appears high.

The relationship of the receptor-endorphin system to tolerance and dependence is still a mystery since all studies to date have proved negative. The existence of model cell culture and organotypic culture systems raises the hope that answers may soon be forthcoming as to whether these phenomena are explicable in terms of changes in endorphin release or in the number or properties of opiate receptors.

There is every reason to believe that future studies in this area will shed light on the mode of action of opiates and will provide insight into the biochemistry underly-

ing analgesia, euphoria, and addiction. It is also likely that these studies will enable investigators to reach a better understanding of the mysteries of the workings of the human and animal brain.

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