OPIOID PEPTIDES AND THEIR RECEPTORS: OVERVIEW AND FUNCTION IN PAIN MODULATION

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Few neurotransmitter systems have fascinated the general public as much as the endorphins, otherwise known as the endogenous opioid peptides. They have been termed the "heroin within" and endowed with the power to relieve pain and allow one to experience "runner's high" or enjoy the taste of chocolate. Although these powers may or may not withstand close scientific scrutiny, there is little question that endogenous opioid systems play a critical role in modulating a large number of sensory, motivational, emotional, and cognitive functions. As inhibitory neuropeptide transmitters, they fine-tune neurotransmission across a wide range of neuronal circuits, setting thresholds or upper limits. In addition, they have served as prototypes for understanding many structural and functional features of peptidergic systems. Thus, the first neuronal receptor binding assays were conducted on opioid receptors. The first peptides to be discovered and identified after the hypothalamic neurohormones (oxytocin and vasopressin) were the endogenous opioids. The first mammalian cyclic DNA (cDNA) to be cloned was an opioid precursor (proopiomelanocortin), which also served as the prototype for genes that encode multiple active substances and process them in a tissuespecific and situation-specific manner.

Scientific studies of these systems during the last 30 years have uncovered a complex and subtle system that exhibits impressive diversity in terms of the number of endogenous ligands (more than a dozen) yet amazing convergence at the level of receptors (only three major types). Based on the results of these studies, the endogenous opioids have been implicated in circuits involved in the control of sensation, emotion, and affect, and a role has been ascribed to them in addiction—not only to opiate drugs, such as morphine and heroin, but also to other highly abused drugs, such as alcohol. This chapter cannot do justice to the rich body of

information we possess on the endogenous opioid system. However, we attempt to give the reader key information about the biochemical nature of the system, along with an update on our understanding of the recently cloned receptors and their functions. Finally, we describe the regulation of pain responsiveness as one example of a function mediated by opioids to illustrate the complexity of their role.

OPIOID PEPTIDES AND THEIR RECEPTORS Genes and Proteins

The opioid peptide precursors are encoded by three genes: pre-proopiomelanocortin, pre-proenkephalin, and pre-prodynorphin. Each precursor is subject to complex post-translational modifications that result in the synthesis of multiple active peptides. These peptides share the common N-terminal sequence of Tyr-Gly-Gly-Phe-(Met or Leu), which has been termed the opioid motif; this is followed by various Cterminal extensions yielding peptides ranging from 5 to 31 residues in length. The major opioid peptide encoded by pre-proopiomelanocortin is β-endorphin. In addition to βendorphin, the proopiomelanocortin precursor encodes the nonopioid peptides adrenocorticotropic hormone (ACTH), α -melanocyte-stimulating hormone (α -MSH), and β -lipotropic pituitary hormone (B-LPH). Pre-proenkephalin encodes multiple copies of Met-enkephalin, including two extended forms of Met-enkephalin (a heptapeptide and an octapeptide), and a single copy of Leu-enkephalin. Pre-prodynorphin encodes three opioid peptides of various lengths that all begin with the Leu-enkephalin sequence: dynorphin A, dynorphin B, and neoendorphin (Fig. 3.1).

The μ -opioid receptors (MORs), δ -opioid receptors (DORs), and κ -opioid receptors (KORs) have been isolated and cloned. The mouse DOR receptor was the first opioid receptor cloned (1,2), and this initial cloning facilitated the rapid cloning of MOR and KOR from various rodent species (3–9). The coding regions of human genes for these

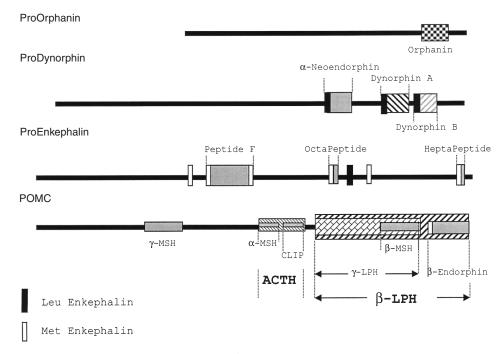


FIGURE 3.1. The opioid-peptide precursors. (From Akil H, Owens C, Gutstein H, et al. Endogenous opioids: overview and current issues. *Drug Alcohol Depend* 1998;51:127–140, with permission.)

receptors were subsequently isolated and chromosomally assigned (10–12). These studies confirmed earlier pharmacologic data indicating that all three receptors belong to the superfamily of seven transmembrane-spanning G protein-coupled receptors. A high degree of structural similarity exists between the three opioid receptors, which is highest in transmembrane domains 2, 3, and 7 and the first and second intracellular loops. The extracellular loops diverge considerably among the three receptor classes, and this divergence may explain differences in ligand selectivity among the opioid receptors (Fig. 3.2).

The relationship between the opioid peptides and their receptors is complex. This has been reviewed in detail elsewhere (13), and we will note only some salient features. It is

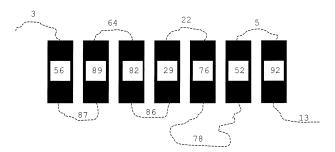


FIGURE 3.2. The opioid receptors display a high degree of structural similarity. Numbers refer to the percentages of amino acid identity between the cloned μ -, δ -, and κ -opioid receptors.

clear from studies of the cloned receptors that high-affinity interactions between each of the precursor and receptor families are possible (14). For example, the proenkephalin peptide Tyr-Gly-Gly-Phe-Met-Arg-Phe binds with subnanomolar affinity to each of the cloned receptors. Similarly, although binding with greater affinity to the KOR, several of the shorter prodynorphin peptides bind with reasonable affinity to the MOR and DOR. By contrast, the binding of shorter proenkephalin peptides Leu-Enk (Tyr-Gly-Gly-Phe-Leu) and Met-Enk (Tyr-Gly-Gly-Phe-Met) readily discriminates between the three receptor families. Overall, the KOR displays the greatest selectivity across the endogenous ligands, with an approximately 1000-fold difference in affinity between the most preferred (Dyn A 1-7) and least preferred (Leu-Enk) ligand, whereas the MOR and DOR differ only across a 10-fold range (14). These differences in selectivity could indicate the existence of distinct mechanisms for ligand recognition, such that MOR and DOR recognize the common Tyr-Gly-Gly-Phe core, whereas the KOR discriminates among the larger variation in C-terminal regions. Indeed, elegant studies in which receptor chimeras were used have identified the critical domains in the three opioid receptors that help discriminate among the endogenous ligands (13).

Further attempts to detect novel opioid receptors resulted in the isolation of a clone with high structural homology to the opioid clones but little or no binding affinity for the opioid ligands (15,16). The structural similarity between this orphan (or opioid receptor-like) opioid receptor

(ORL-1) and the opioid receptors is highest in the transmembrane regions and cytoplasmic domain and lowest in the extracellular domains critical for ligand selectivity (see below). A ligand for the receptor was subsequently identified by two groups using chromatographic fractionation techniques coupled to ORL-1-mediated inhibition of adenylyl cyclase (17,18). This 17-amino acid peptide is identical in length and C-terminal sequence to dynorphin A. Curiously, the N-terminal is slightly modified (Phe-Gly-Gly-Phe) from the opioid core described above. It has been termed orphanin-FQ or nociceptin because of its putative ability to lower pain thresholds. The orphanin-FQ/nociceptin (OFQ/N) precursor has been cloned from mouse, rat, and human and has been localized on the short arm of human chromosome 8 (19,20). In addition to OFQ/N, evidence suggests that this precursor may encode other biologically active peptides. Immediately downstream to OFQ/ N is a 17-amino acid peptide (OFQ-2) that also starts with phenylalanine and ends with glutamine but is otherwise distinct from OFQ/N, and a putative peptide upstream from OFQ/N may be liberated on post-translational processing (nocistatin). The OFQ/N system is a distinct neuropeptide system with a high degree of sequence identity to the opioids. This slight change in structure results in a profound alteration in function. Thus, OFQ/N is motivationally neutral, as indexed by conditioned place preference (21), and has pain modulators distinct from those of the opioid peptides (see below). However, changes in as few as four amino acids endow the ORL-1 receptor with the ability to recognize prodynorphin products while still retaining recognition of OFQ/N (22). These findings suggest that unique mechanisms may have evolved to ensure selectivity against the opioids versus selectivity for OFQ/N.

Issues and Complexities Revealed by Multiple Pharmacologic Forms, Splice Variants, and Receptor Dimers

The molecular cloning studies described above identified only a single gene encoding each of the opioid receptors. These findings contrast with results of pharmacologic studies indicating the existence of two subtypes of MOR and DOR and up to four subtypes of KOR, and suggesting additional receptor families (23–26). For example, the DOR₁ subtype is held to display greater affinity for the agonist DPDPE, whereas the DOR2 subtype displays greater affinity for the agonist deltorphin 2 (26). These two subtypes may also make independent contributions to DOR antinociception (27). It is possible that further molecular cloning will identify unique genes encoding these receptor subtypes. However, several authors have suggested that if multiple opioid-receptor subtypes exist, they could be derived from a single gene, and multiple mechanisms might exist to achieve these distinct pharmacologic profiles. In this section, we consider two such pathways to opioid-receptor diversity: alternative splicing of receptor RNA and dimerization of receptor proteins.

Alternative splicing of receptor heteronuclear RNA (e.g., exon skipping and intron retention) has been accorded an important role in producing in vivo diversity within many members of the superfamily of seven transmembrane-spanning receptors (28). For example, alternative splicing of the coding region for the N-terminus of the corticotropin-releasing hormone CRH-2 receptor results in α , β , and γ variants, each with a unique tissue distribution (see ref. 28 for review). It follows that splice variants may exist within each of the three opioid-receptor families and that this alternative splicing of receptor transcripts may be critical for the diversity of opioid receptors. A technique used extensively to identify potential sites of alternative splicing is antisense oligodeoxynucleotide (ODN) mapping. The ability of antisense ODNs to target specific regions of cDNA allows systematic evaluation of the contribution of individual exons to the observed properties of a receptor. Antisense ODN targeting of exon 1 of the cloned rat and mouse MOR prevents morphine analgesia in these species (29–31). By contrast, administration of antisense ODNs targeting exon 2, which are inactive against morphine analgesia, prevents the analgesia produced by heroin, fentanyl, and the morphine metabolite morphine-6-β-glucuronide (M6G) (29-31). A similar disruption of M6G but not morphine analgesia is observed following administration of antisense ODNs targeting exon 3 (29). These results suggest that unique MOR mechanisms may mediate the analgesic effects of a variety of opiate alkaloids, and are consistent with the claim that these unique receptor mechanisms could be achieved via alternative splicing. The use of antisense ODNs has also resulted in the identification of potential sites for splice variation in the KOR and DOR (32). Central to the claim that these results reflect the existence of opioid-receptor splice variants is the in vivo isolation of such variants. A splice variant of the MOR has been identified that differs considerably within its C-terminus (33). As might be expected on the basis of the location of the alternative splicing, this variant exhibits a binding profile similar to that of the cloned MOR but does not readily undergo the desensitization frequently observed following agonist exposure. Thus, although it differs in composition, the existence of this splice variant cannot explain the results described above. However, just such a variant was detected in mice subjected to targeted disruption of exon 1 (34). Thus, transcripts of the MOR that contained exons 2 and 3 were identified in exon 1deficient mice. Moreover, whereas morphine analgesia was abolished, heroin and M6G analgesia was retained in these mice (see below).

The physical interaction of receptors to form a unique structure (dimerization) has also been accorded an important role in regulating receptor function. For example, dimerization of GABA_BR1 (γ -aminobutyric acid subtype B receptor 1) and GABA_BR2 subunits is required for the for-

mation of a functional GABA_B receptor (35). Both the cloned KORs and DORs have been found to exist in vitro as homodimers (36). However, the most important demonstrations of this kind are those showing dimerization between different functional opioid-receptor types. Jordan and Devi (37) coexpressed tagged KOR and DOR or tagged KOR and MOR and used coprecipitation techniques to show that KOR and DOR can exist as heterodimers in vitro. Dimerization of these receptors profoundly altered their properties. The affinity of the heterodimers for highly selective KOR and DOR agonists and antagonists was greatly reduced. Instead, the heterodimers showed greatest affinity for partially selective agonists such as bremazocine. This pharmacologic profile is similar to that claimed for the KOR2-receptor subtype, which suggests that receptor dimerization may explain at least part of the discrepancy between the molecular and pharmacologic properties of opioid receptors. Heterodimerization thus offers a mechanism for the formation of novel receptor forms and a possible explanation for the in vivo diversity of opioid receptors. It will be of particular interest to identify those factors governing formation of opioid-receptor heterodimers, to determine if and how frequently opioid receptors dimerize in vivo, and to generate ligands that selectively recognize the dimerized form of the receptors.

Signal Transduction Mechanisms and Their Adaptation after Chronic Stimulation

The opioid receptors couple to G proteins inhibiting adenylyl cyclase, activating inwardly rectifying K⁺ channels and decreasing the conductance of voltage-gated Ca²⁺ channels (38). These mechanisms of signal transduction have been verified by studies of the cloned receptors expressed in a variety of host cells (see ref. 39 for review). Studies with the cloned receptors have also indicated that the opioid receptors may couple to an array of other second messenger systems, which include activation of the mitogenactivated protein (MAP) kinases and the phospholipase Cmediated cascade leading to the formation of IP₃ (inositol-1,4,5-triphosphate) and diacyl glycerol (see ref. 40 for review). Prolonged exposure to opiates results in adaptations at multiple levels within these signaling cascades. The significance of these adaptations rests, at least in part, in the causal relationship that may exist between them and those seen at the organismic level following iterated exposure to opiates, such as tolerance, sensitization, and withdrawal. We consider two such adaptations: those related to cyclic adenosine monophosphate (cAMP) signaling and those related to receptor densensitization and internalization.

In an elegant series of experiments using rat locus ceruleus (LC) neurons, Nester and co-workers identified increased levels of protein $G_{i\alpha}$ and protein $G_{o\alpha}$, adenylyl cyclase, and protein kinase A following prolonged *in vivo*

exposure to morphine (41-43). This up-regulation of the cAMP signaling pathway mediates the increased excitability of LC neurons observed after prolonged exposure to morphine and has been invoked as a causal mechanism for the increased or "rebound" activity of LC neurons frequently observed when the drug is withdrawn (44,45). It follows that these compensatory adaptations in cAMP signaling could be causal to the opiate withdrawal syndrome observed at the organismic level. Consistent with this possibility, infusions of a protein kinase A inhibitor into the LC reduced the severity of the antagonist-precipitated withdrawal syndrome in rats (46,47). Importantly, these adaptations are not unique to opioid receptors in the LC. Increased levels of adenylyl cyclase and protein kinase A in response to chronic morphine exposure have also been detected in the nucleus accumbens and amygdala (48). However, the changes in levels of G-protein subunits induced by this treatment are more complex, with decreased levels of $G_{i\alpha}$ detected in the nucleus accumbens and increased levels of Gia and Gio in the amygdala. These widespread changes are also of significance at the organismic level. For example, infusions of a protein kinase A inhibitor into the periacqueductal gray (PAG) reduced the severity of the antagonist-precipitated withdrawal syndrome (47), and inactivation of G_i and G_o proteins in the nucleus accumbens reduced heroin self-administration in rats (49).

The desensitization, internalization, and sequestration of opioid receptors following their activation may also constitute mechanisms for adaptation in signaling relevant for understanding alterations in the physiologic impact of opiates. For example, phosphorylation of MOR and DOR via protein kinase C results in a transient desensitization that could subserve acute tolerance to opiates (50–53). Similarly, the internalization of opioid receptors via a classic endocytic pathway may have important implications for the physiologic impact of opiates. The internalization of opioid receptors occurs in a ligand-specific manner. For example, DAMGO and methadone promote internalization of the MOR, but morphine does not (53,54). This ligand-specific internalization is determined, at least in part, by differences in the conformational changes induced by the ligand and is independent of its ability to stimulate G-protein signaling (55). These findings may offer a novel explanation of differences in the efficacy and abuse potential of various opiates. However, at the time of this writing, few attempts have been made to study the relevance of these alterations in signaling to the adaptations seen in response to opiate exposure in vivo. Perhaps the most interesting are demonstrations that acute morphine analgesia is enhanced in mice in which the gene encoding β -arrestin 2 was disrupted (56). Opioid-receptor internalization is mediated, at least in part, by the actions of the G protein-receptor kinases (GRKs). The GRKs selectively phosphorylate the agonist-bound receptor promoting interactions with β-arrestins, which interfere with G-protein coupling and promote receptor internalization (56). Demonstrations that acute morphine analgesia is enhanced in mice lacking β -arrestin 2 are consistent with a role for the GRKs and arrestins in regulating alterations in responsivity to opiates *in vivo*. This finding is even more intriguing given the inability of morphine to support arrestin translocation and receptor internalization *in vitro* (57).

RECEPTOR AND LIGAND KNOCKOUTS: INSIGHTS, ISSUES, AND COMPLEXITIES

Advances in understanding the molecular biology of the opioid family, coupled with developments in recombinant technology, have resulted in the generation of mice with targeted disruptions of various opioid genes. The study of these animals offers unique insights into opioid-receptor function. The initial study of these mice has allowed evaluation of the critical receptor subtypes mediating the effects of a variety of opiate alkaloids and the selective peptide agonists. In addition, they have identified potential interactions between receptor subtypes and suggested novel functions for opioids (e.g., reproductive function).

MOR Knockouts

The MOR gene has been disrupted via targeted deletion of exon 1 (34,58,59), exon 2 (34), or exons 2 and 3 (60). Disruption of exons 2 and 3 had no detectable effect on development, health, and fertility (60), whereas disruptions of exon 1 impaired sexual function in male mice, manifested by reduced mating activity, decreased sperm count and motility, and smaller litter size (59). Evidence was also found for alterations in hematopoiesis—specifically, increased proliferation of granulocyte-macrophages and erythroid and multipotential progenitor cells—in exon 1 knockout mice (59). Assessment of these mice has revealed that the MOR is absolutely necessary for the analgesic effects of morphine. Thus, systemic, intracerebal ventricular, and intrathecal administration of morphine failed to produce analgesia as assayed by tail flick, hot plate, and paw withdrawal tests across a wide dose range. For example, doses of morphine as high as 56 mg/kg failed to produce analgesia in exon 1 knockout mice (58), and the median effective dose (ED₅₀) for morphine analgesia in exon 2 knockout mice exceeded 100 mg/ kg (a potency shift of two orders of magnitude) (34). The MOR is also required for the rewarding (indexed by levels of conditioned place preference) and immunosuppressive effects of injections of morphine, and for the physical dependence induced by such injections (indexed by somatic signs of morphine withdrawal) (60,61). By contrast, the analgesic efficacy of heroin and the major morphine metabolite M6G remains intact in exon 1-deficient mice (34). This result is consistent with the antisense mapping studies described above. Although successfully identifying the critical receptor substrate for the therapeutic and recreational

uses of morphine, these experiments have failed to address the involvement of MOR in basal pain sensitivity convincingly. For example, considerable controversy has surrounded the ability of an injection of naloxone to produce hyperalgesia in otherwise intact animals, and this has not been resolved by studies of the MOR knockout mice. Sora et al. (58) reported that MOR knockout mice displayed increased sensitivity to noxious stimulation, but this hyperalgesia was not readily detected by others (60). This difference could be related to differences in the impact of specific exon deletion, as in measurements of reproductive function. Alternatively, a stress-induced analgesia, such as that provoked by exposure to novel handling procedures or contextual cues, may have decreased basal pain sensitivity among control animals.

In addition to providing insight into the mechanisms of actions of the opiate alkaloids, studies of MOR knockout mice have allowed systematic investigation into the potential interactions between the three opioid-receptor families in vivo. Studies of DOR function in MOR knockout mice have failed to detect compensatory changes in either the number or localization of DORs (62). Similarly, no significant alteration in DOR signal transduction, as indexed by G-protein and adenylyl cyclase activity, has been observed (63). By contrast, the analgesic efficacy of DOR agonists in these mice may be slightly reduced. Specifically, a reduction in DPDPE analgesia appears most robust, whereas the analgesic effects deltorphin 2 have been found intact or slightly attenuated (63,64). This evidence for MOR-mediated effects of DOR agonists is intriguing and consistent, at least in part, with the possibility of interactions between MOR and DOR in vivo. However, it is worth bearing in mind that these studies uniformly indicate the preservation of a large component of DOR function in MOR knockout mice. Studies of KOR function in MOR knockout mice have also failed to detect significant alterations in receptor number, distribution, and signal transduction (62,63). However, no evidence has been found of a reduction in the analgesic efficacy of KOR agonists, unlike that of DOR agonists, in MOR knockout mice (64).

DOR Knockouts

The *DOR* gene has been disrupted in mice via targeted disruption of exon 2 (65). This deletion had no detectable effects on the health or reproductive function of the mice. Deletion of exon 2 completely abolished [³H]DPDPE and [³H]deltorphin 2 binding in the brain, which indicates that the putative subtypes of the DOR are encoded by the same gene product. Studies of pain sensitivity in these mice indicate that basal pain sensitivity is unaffected by disruption of the *DOR* gene. Spinal DPDPE and deltorphin 2 analgesia is significantly reduced in the DOR knockout mice. By contrast, the analgesic efficacy of intracerebral ventricular infusions of DPDPE and deltorphin 2 remains intact. The

retention of supraspinal but not spinal DOR analgesia in DOR knockout mice is surprising. This could be evidence for a novel receptor mechanism because this residual supraspinal analgesia is reduced by naltrexone but not by selective MOR or KOR antagonists. Disruption of the DOR gene has no significant effect on the levels and distribution of either MOR or KOR, nor is any effect noted on the levels and distribution of proenkephalin, prodynorphin, and proopiomelanocortin. Similarly, no significant alterations occur in the analgesic effectiveness of morphine, M6G, and the κ agonist U50,488H.

KOR Knockouts

The KOR gene has been disrupted in mice via targeted deletion of the initiation codon and N-terminal coding region (66). This disruption had no detectable effects on the health of the mice but increased litter size. The deletion completely abolished [3H]CI-977 binding in the brain. Studies of pain sensitivity revealed that KOR knockout mice are hyperalgesic when assayed by the acetic acid writhing test but not the formalin, tail pressure, tail flick, and hot plate tests. This finding is consistent with the important role accorded KOR in the regulation of visceral nociception. Systemic injection of the KOR agonist U50,488H failed to produce an analgesic response as assayed by the tail flick and hot plate tests. Similarly, the locomotor depressive effects and aversive motivational effects of the injection (indexed by conditioned place aversion learning) were abolished. These results indicate that the analgesic and motivational effects of the prototypical KOR agonist are mediated via actions at the receptor(s) encoded by the KOR gene and are consistent with results of antisense mapping studies indicating that ODNs directed against each of the three exons of the KOR gene disrupt the analgesic efficacy of U50,488H. The effects of disruption of the KOR gene on the activity of dynorphin B and α -neodynorphin, whose selectivity in antisense mapping studies differs considerably from that of U50,488H (67), remains unclear. Disruption of the KOR gene had no significant effect on the levels and distribution of either MOR or DOR (68), nor was any effect noted on the level and distribution of proenkephalin, prodynorphin, and proopiomelanocortin (67). Interestingly, the analgesic efficacy of morphine was retained, but the aversive motivational effects of the dependence induced by iterated exposures to morphine were reduced. This finding supports demonstrations of a role for dynorphin and the KOR in opiate withdrawal (69).

Pre-proenkephalin Knockouts

Mice with targeted deletions of exon 3 of the pre-proenkephalin gene have been created (70). Although this disruption had little effect on levels of prodynorphin- and proopiomelanocortin-derived peptides, a large up-regulation of

MOR binding in the striatum was observed (71). Neither fertility nor gross abnormalities developed in the enkephalin knockout animals. These mice displayed increased anxiety and fear-related behaviors (indexed by freezing, hiding, and performance in an open field and elevated O maze). These results suggest that enkephalins are important in the negative feedback control of anxiety and aversive motivation. Enkephalin knockout mice appeared hyperalgesic when tested with the hot plate, but not the tail flick, test. However, because the procedure for this test involved repeated exposure to the hot plate apparatus, it is again unclear whether the experimental mice were hyperalgesic or whether the control mice were hypoalgesic as a consequence of repeated testing in the hot plate apparatus. The enkephalin knockout mice also showed altered sensitivity when assayed by the formalin test. Specifically, a decrease in recuperative behaviors (lifting and licking the injected paw) could be mimicked by injection of naloxone (10 mg/kg) in wild-type control mice, which suggests that the proenkephalin-derived peptides may regulate responding in the formalin test. This result is also difficult to interpret because naloxone does not modulate formalin pain in rats under resting conditions. In short, across three measures of pain sensitivity, three different influences of the deletion of the pre-proenkephalin gene were detected: no effect in the tail flick test, increased sensitivity (hyperalgesia) in the hot plate test, and decreased sensitivity (indexed by recuperative responding) in the formalin test. Although dissociations between these measures are not uncommon, the pattern of responding across the three measures is difficult to interpret and underscores the complexity of pain modulation by aversive motivational states such as anxiety and fear. Indeed, these mice displayed intact analgesic responses to stressors (swim stress) that produce naloxone-reversible analgesia. This result is consistent with the binding studies reviewed above, indicating the potential for high-affinity interactions between peptides derived from the proopiomelanocortin and prodynorphin precursors and each receptor class.

Orphanin-FQ-Receptor and Ligand Knockouts

The gene encoding the ORL-1 receptor has been disrupted via targeted deletion of exon 1 (72), and the OFQ/N precursor has been disrupted via targeted deletion of exon 2 (73). Studies of these mice have proved particularly interesting. First, they have confirmed that the role of OFQ/N in pain modulation is quite distinct from that of the other opioids. Thus, disruption of the ORL-1 receptor had no effect on basal pain sensitivity in the tail flick test but prevented the development of tolerance to morphine analgesia (72), whereas disruption of the OFQ/N precursor consistently decreased pain sensitivity on the same measure. This discrepancy between the effects of receptor and precursor disruption could be interpreted to mean that post-translational

processing of the OFQ/N precursor may result in the presence of multiple active peptides that interact with unique receptors to produce different physiologic effects (see above). However, these studies uniformly indicate that if OFQ/N and the ORL-1 receptor have any role in pain modulation, it is facilitative (or pronociceptive) rather than inhibitory (or antinociceptive). Second, these studies have confirmed that OFQ/N serves an important role in the regulation of emotional responsiveness. Specifically, OFQ/N knockout mice display increased anxiety (indexed by performance in the elevated plus maze, open field, and light-dark box) and enhanced basal and post-stress glucocorticoid levels. Interestingly, these findings contrast with the effects of administration in rats. Devine et al. (74) have shown that infusions of OFQ/N increase plasma ACTH and glucocorticoid levels in the unstressed animal and prolong the stress response in the stressed rat. The reasons for this discrepancy are unclear. Finally, these studies have suggested an important role for the OFQ/N system in learning and memory processes. Thus, OFQ/N-receptor knockout mice show enhanced hippocampal long-term potentiation and a moderately enhanced performance in tests of spatial learning (75). However, the OFQ/N-precursor knockout mice do not show enhanced performance in the same test of spatial learning (73). Regardless of the reason for the discrepancy between the OFQ/N and ORL-1 knockout mice, the interpretation of these effects on learning and memory is difficult. For example, the spatial task used in these experiments can be mediated by several learning strategies. Clearly, a more sophisticated characterization of the nature of the potential learning and memory deficits in these mice is required, and the results described above provide an important starting point.

Summary

Studies of mice with targeted disruptions of opioid-receptor and peptide genes have enabled important insights into the function of the opioid family. Chiefly, they have made possible the identification of the critical receptor substrates for a variety of opiate alkaloids and opioid peptides. These studies have also provided insights into the functional diversity of each receptor class. For example, it is clear that the two subtypes of DOR identified in pharmacologic studies are encoded by the single cloned DOR gene. Furthermore, the retention of MOR- and KOR-independent supraspinal DPDPE analgesia in these mice raises the possibility that further, unidentified opioid-receptor variants may exist. A similar possibility is raised by the retention of heroin and M6G analgesia in mice with targeted deletions of the MOR. These results suggest that complex post-transcriptional modifications play an important role in producing the in vivo diversity of opioid-receptor pharmacology. At the time of this writing, only OFQ/N-receptor and OFQ/N-precursor knockout mice have been studied in more complex be-

havioral tasks. However, the widespread distribution of opioid peptides and their receptors in the central nervous system, in addition to their critical role in controlling an animal's interaction with its environment, ensure that it is only a matter of time before mice are studied with more behaviorally sophisticated and ecologically relevant measures of attention, learning, memory, and motivation. Finally, it is worth noting that this first generation of genetic manipulations are neither tissue-specific nor conditional. Compensatory adaptation within the opioid-peptide and receptor family following the targeted disruption of one of its members appears to be minimal. Indeed, in the studies reviewed above, the only evidence for such compensation has been obtained for measures of receptor binding in preproenkephalin knockout animals. Nonetheless, the possibility of widespread adaptation in nonopioid systems cannot be discounted. Thus, the application of tissue-specific and inducible knockout techniques to the opioid receptors and their peptides remains an exciting area of research.

AN EXAMPLE OF OPIOID FUNCTION AND ITS CLINICAL IMPLICATIONS: OPIOIDS AND PAIN CONTROL

Understanding the role of opioids in pain modulation is not only of clinical importance but also of historical interest. Demonstrations that microinjections of morphine into various brainstem regions are analgesic (76), and that injections of naloxone partially reverse the analgesia produced by focal electric stimulation in these regions (77,78), provided the first physiologic evidence for an endogenous opioid system. In this section, we briefly review the neural circuits subserving opioid analgesia and discuss recent findings relevant to these actions. Many excellent reviews of this topic are available (79,80).

Functional Anatomy of Opioids in Descending Pain Control Circuits

It is well established that the analgesic effects of opioids arise from their ability to inhibit directly the ascending transmission of nociceptive information from the spinal cord dorsal horn, and from their ability to activate pain control circuits that descend from the midbrain, via the rostral ventromedial medulla (RVM), to the spinal cord dorsal horn. Opioid peptides and their receptors are distributed throughout these descending pain control circuits (81, 82). MOR messenger RNA (mRNA) or binding has been detected throughout the PAG, pontine reticular formation, median raphe, nucleus raphe magnus and adjacent gigantocellular reticular nucleus in the RVM, and spinal cord. Inspection of the discrepancies between levels of receptor binding and mRNA expression provide important insights into the mechanisms of MOR analgesia. For example, the

presence of significant MOR binding in the superficial dorsal horn but scarcity of mRNA expression suggests that the majority of these spinal MOR binding sites are located presynaptically on the terminals of primary afferent nociceptors. This conclusion is consistent with the high levels of MOR mRNA expression in dorsal root ganglia (DRG). A similar mismatch between MOR binding and mRNA expression can be found in the dorsolateral PAG (strong binding vs. sparse mRNA). DOR mRNA and binding have been detected in the ventral and ventrolateral quadrants of the PAG, pontine reticular formation, and gigantocellular reticular nucleus, but only at low levels in the median raphe and nucleus raphe magnus. Like MOR binding sites, DOR binding sites are present in significant numbers in the dorsal horn without detectable mRNA expression, which suggests an important role for presynaptic actions of DOR in spinal analgesia. Finally, KOR mRNA and binding are widely distributed throughout the PAG, pontine reticular formation, median raphe, and nucleus raphe magnus and adjacent gigantocellular reticular nucleus. Again, significant levels of KOR binding but sparse levels of mRNA have been found in the dorsal horn. Although all three receptor mRNAs are found in the DRG, they are localized on different classes of primary afferent nociceptors. Thus, MOR mRNA has been detected in medium- and large-diameter DRG cells, DOR mRNA in large-diameter cells, and KOR mRNA in small- and medium-diameter cells. This differential localization could be linked to functional differences in pain modulation.

The distribution of opioid receptors in descending pain control circuits indicates substantial overlap between MOR and KOR. The largest differentiation between these two receptors and DOR is in the PAG, median raphe, and nucleus raphe magnus (82). A similar differentiation of MOR and KOR from DOR is observed in the thalamus, which suggests that interactions between KOR and MOR may be important for modulating nociceptive transmission from the dorsal horn as well as in higher nociceptive centres. The actions of MOR agonists are invariably antinociceptive, whereas those of KOR agonists can be either antinociceptive or pronociceptive. Consistent with the anatomic overlap between the MOR and KOR, the pronociceptive actions of the KOR appear to be mediated by a functional antagonism of the actions of the MOR. The MOR produces antinociception within descending pain control circuits, at least in part, via the removal of GABAergic inhibition of RVM projecting neurons in the PAG and spinally projecting neurons in the RVM (79). Pan et al. (83) have presented evidence from both in vitro slice preparations and in vivo pain responding that the pain modulatory effects of the KOR in the brainstem oppose those of the MOR. Thus, activation of the KOR hyperpolarized the same RVM neurons hypopolarized by the MOR, and microinjections of a k agonist into the RVM antagonized the analgesia produced by microinjections of DAMGO into this region. These data are among the strongest that opioids can have pronociceptive in addition to antinociceptive effects and could explain behavioral evidence for a reduction in hyperalgesia following injections of naloxone.

As described above, significant opioid-receptor binding, little detectable expression of receptor mRNA in the spinal cord dorsal horn, but large levels of this mRNA in DRG have been observed. The anatomy of spinal opioid receptors suggests that their actions relevant to analgesia at this level are predominantly presynaptic. At least one presynaptic mechanism viewed as having clinical significance is the inhibition of spinal tachykinin signaling. Indeed, it is well established that opioids decrease the noxious stimulant-evoked release of tachykinins from primary afferent nociceptors (84, 85). Recently, the significance of this action has been questioned. Measuring the internalization of neurokinin receptors following noxious stimulation, Trafton et al. (86) demonstrated that at least 80% of tachykinin signaling remains intact after the intrathecal administration of large doses of opioids. These results indicate that although opioid administration may reduce tachykinin release from primary afferent nociceptors, the reduction has little functional impact on the actions of tachykinins on postsynaptic nociceptive neurons. The obvious implication of this finding is that either tachykinin signaling is not central to nociception and/ or opioid antinociception at the spinal level, or that, contrary to the conclusions suggested by anatomic studies, the presynaptic actions of opioids are of little analgesic significance.

Just as important insights have been made into brainstem and spinal mechanisms for opioid analgesia, so too have insights been made into forebrain mechanisms for such analgesia. It is well established that the actions of opioids in bulbospinal pathways are critical to their analgesic efficacy. It has been less clear what role should be accorded forebrain actions and whether these actions are independent of those in bulbospinal pathways. There can be little doubt that opioid actions in the forebrain contribute to analgesia because decerebration prevents analgesia when rats are tested for pain sensitivity with the formalin test (87), and microinjections of opioids into the several forebrain regions are analgesic in this test (88). However, because these manipulations frequently leave intact the analgesic efficacy of opioids in measures of phasic nociception, such as the tail flick test, a distinction has been drawn between forebrain-dependent mechanisms for morphine analgesia in the presence of tissue injury and bulbospinal mechanisms for this analgesia in the absence of tissue injury. In an important series of experiments, Manning and Mayer (89,90) have shown that this distinction is not absolute and that opioid actions in the forebrain are also important to analgesia, both in measures of tissue damage and in acute, phasic nociception. Thus, systemic morphine analgesia in both the tail flick and formalin tests was disrupted by either lesioning or reversible inactivation of the central nucleus of the amygdala. The involvement of the amygdala in morphine analgesia is particularly interesting because this structure has been implicated in the environmental activation of pain control circuits, and it projects extensively to those brainstem regions involved in descending pain control (80).

Role of OFQ/N and ORL-1 in Pain Modulation

OFQ/N mRNA and peptide are present throughout the descending pain control circuits described above. For example, OFQ/N-containing neurons are present in the PAG, the median raphe, throughout the RVM, and the superficial dorsal horn (91) This distribution overlaps with that of the opioid peptides, but the degree of colocalization remains unclear. ORL-1 binding and mRNA can be detected in the PAG, median raphe, and RVM (92). In the spinal cord, ORL-1 mRNA expression is stronger in the ventral than in the dorsal horn, but levels of binding are higher in the dorsal horn. High levels of ORL-1 mRNA are also found in the DRG. Despite this clear anatomic evidence for a role of the orphanin system in pain modulation, its function remains unclear. As reviewed above, targeted disruption of the ORL-1 receptor had little effect on basal pain sensitivity according to several measures, whereas targeted disruption of the OFQ/N precursor consistently elevated pain sensitivity in the tail flick test, findings that suggest an important role for OFQ/N in regulating basal pain sensitivity. Intrathecal injections of OFQ/N have been reported to be analgesic as assayed by the tail flick and formalin tests (93,94). Similarly, these injections attenuated the hyperalgesia produced by constriction injury of sciatic nerve (95). However, the profound motor effects of these injections render interpretation of changes in response latency difficult. The effects of supraspinally administered OFQ/N are also difficult to interpret; hyperalgesia has been detected across a variety of measures, but failures to detect hyperalgesia have also been reported.

Three interesting results may explain at least part of the variations noted in the effects of the orphanin opioid system in modulating pain. First, Rossi et al. (95,96) reported a biphasic effect of OFQ/N administration, characterized initially by hyperalgesia and later by analgesia. Second, Grisel et al. (97) reported that OFQ/N does not affect basal pain sensitivity but does reduce analgesia according to the site of administration. Finally, Heinricher et al. (98) reported that OFQ/N exerts an inhibitory effect on several classes of RVM neurons whose activity has been implicated in producing analgesia and hyperalgesia at the spinal level. These results suggest that the effects on pain modulation observed following administration of OFQ/N in the intact animal are influenced by route, time since administration, the presence of stressors that provoke analgesia (e.g., novel handling or test procedures), and the current balance of activity in pain modulatory neurons in the RVM. The development of specific ORL-1-receptor antagonists will undoubtedly enable a rapid clarification of the role of the orphanin opioid system in pain modulation.

CONCLUSIONS AND FUTURE DIRECTIONS

The interplay between the orphanin system and the endogenous opioids represents a prime example of evolutionary changes that have led to subtle diversity in structure and significant alteration in function. Indeed, this entire peptidergic family exemplifies the way in which an increase in genetic diversity can lead from simple on/off signaling to a complex pattern of signaling wherein multiple, coordinately secreted peptides interact with multiple receptors to effect a complex regulation of functions as diverse as pain responsiveness, stress regulation, control of feeding, and modulation of development, learning, and memory. Many questions remain to be answered in the context of the opioid family. At the most basic level is the question of whether additional members of the family exist. The completion of sequencing of the human genome and the rat or mouse genome should help answer this question. We should be able to lay to rest the questions of whether additional opioidreceptor types or subtypes exist, and whether other endogenous ligands that are uniquely selective for a particular receptor type exist. In particular, endomorphin 1 (Tyr-Pro-Trp-Phe) and endomorphin 2 (Tyr-Pro-Phe-Phe) have been proposed by Zadina et al. (99) to be endogenous, highly selective µ ligands. However, their precursor remains uncloned, although the genome project should help clarify the matter. Further, as we obtain full sequences of the genomes of other species, we should be able to track the fascinating evolutionary history of this peptide family.

At functional levels, many questions remain, especially concerning the exact role of endogenous opioids in addictive and emotional behavior and psychiatric disorders. Because these disorders are typically of a complex genetic nature, involving the interaction of multiple genes with one another and with the environment, it is likely that the endogenous opioid genes are involved in vulnerability to certain brain-related illnesses. Here again, progress in genomics and complex genetics should open new avenues for investigating the likely role of the opioid molecules in a range of psychiatric disorders.

REFERENCES

- Evans CJ, Keith DE, Morrison H, et al. Cloning of a delta opioid receptor by functional expression. Science 1992;258:1952–1955.
- Kieffer B, Befort K, Gaveriaux-Ruff C, et al. The delta opioid receptor: isolation of a cDNA by expression cloning and pharmacological characterization. *Proc Natl Acad Sci U S A* 1992;89: 12048–12052.
- Chen Y, Mestek A, Liu J, et al. Molecular cloning and functional expression of a μ-opioid receptor from rat brain. Mol Pharmacol 1993;44:8–12.

- 44
- Kong, H, Raynor R, Yano H, et al. Agonists and antagonists bind to different domains of the cloned kappa receptor. *Proc Natl Acad Sci U S A* 1994;91:8042–8046.
- Meng F, Xie GX, Thompson RC, et al. Cloning and pharmacological characterization of a rat kappa opioid receptor. *Proc Natl Acad Sci U S A* 1993;90:9954–9958.
- Minami M, Toya T, Katao Y, et al. Cloning and expression of a cDNA for the rat κ-opioid receptor. FEBS Lett 1993;329: 291–295.
- Thompson RC, Mansour A, Akil H, et al. Cloning and pharmacological characterization of a rat mu-opioid receptor. *Neuron* 1993;11:903–913.
- Wang JB, Imai Y, Eppler CM, et al. μ Opiate receptor: cDNA cloning and expression. Proc Natl Acad Sci U S A 1993;90: 10230–10234.
- Yasuda K, Raynor K, Kong H, et al. Cloning and functional expression comparison of kappa and delta opioid receptors from mouse brain. *Proc Natl Acad Sci U S A* 1993;90:6736–6740.
- Befort K, Mattei MG, Roeckel N, et al. Chromosomal localization of the δ opioid receptor gene to human 1p34.3-p36.1 and mouse 4D bands by *in situ* hybridization. *Genomics* 1994;20: 143–145.
- Yasuda K, Espinoza R, Takeda J, et al. Localization of the kappa opioid receptor gene to human chromosome band 8q11.2. *Geno*mics 1994;19:596–597.
- Wang JB, Johnson PS, Perisco AM, et al. Human μ-opiate receptor cDNA and genomic clones, pharmacologic characterization and chromosomal assignment. FEBS Lett 1994;338:217–222.
- 13. Akil H, Owens C, Gutstein H, et al. Endogenous opioids: overview and current issues. *Drug Alcohol Depend* 1998;51:127–140.
- Mansour A, Hoversten M, Taylor LP, et al. The cloned μ, δ, or κ receptors and endogenous ligands: evidence for two opioid peptide recognition cores. *Brain Res* 1995;700:89–98.
- 15. Bunzow JR, Saez C, Mortrud M, et al. Molecular cloning and tissue distribution of a putative member of the rat opioid receptor gene family that is not a μ , δ , or κ receptor type. *FEBS Lett* 1994;347:279–282.
- Mollereau C, Parmentier M, Mailleux P, et al. ORL1, a novel member of the opioid receptor family—cloning, functional expression and localization. FEBS Lett 1994;341:33–38.
- Meunier JC, Mollereau C, Toll L, et al. Isolation and structure of the endogenous agonist of the opioid receptor like ORL₁ receptor. *Nature* 1995;377:532–535
- Reinscheid RK, Nothacker HP, Bourson A, et al. Orphanin FQ: a neuropeptide that activates an opioid-like G-protein-coupled receptor. *Science* 1995;270:792–794.
- 19. Nothacker HP, Reinscheid RK, Mansour A, et al. Primary structure and tissue distribution of the orphanin FQ precursor. *Proc Natl Acad Sci U S A* 1996;93:8622–8677.
- Pan YX, Xu J, Pasternak GW, Cloning and expression of a cDNA encoding a mouse brain orphanin FQ/nociceptin precursor. *Bio-chem J* 1996;315:11–13.
- Devine DP, Reinscheid RK, Monsma FJ, et al. The novel neuropeptide orphanin FQ fails to produce conditioned place preference or aversion. *Brain Res* 1996;727:225–229.
- 22. Meng F, Taylor LP, Hoversten M, et al. Moving from the orphanin FQ receptor to an opioid receptor using four point mutations. *J Biol Chem* 1996;271:32016–32020.
- Zukin RZ, Eghali M, Olive D, et al. Characterization and visualization of rat and guinea pig brain κ opioid receptors: evidence for κ1 and κ2 opioid receptors. *Proc Natl Acad Sci U S A* 1998; 85:4061–4065.
- Paul D, Bodnar RJ, Gistrak MA, et al. Different μ receptor subtypes mediate spinal and supraspinal analgesia in mice. Eur I Pharmacol 1989;168:307–314.
- 25. Sofuoglu M, Portoghese PS, Takemori AE. Differential antago-

- nism of δ opioid agonists by naltrindole and its benzofuran analog (NTB) in mice: evidence for δ opioid receptor subtypes. *J Pharmacol Exp Ther* 1991;257:676–680.
- Zaki PA, Bilsky EJ, Vanderah TW, et al. Opioid receptor types and subtypes: the δ receptor as a model. Ann Rev Pharmacol Toxicol 1996;36:379–401.
- Stewart PE, Hammond DL. Evidence for δ opioid receptor subtypes in rat spinal cord: studies with intrathecal naltriben, cyclic (D-Pen², D-Pen⁵)-enkephalin and (D-Ala², Glu⁴)-deltorphin. J Pharmacol Exp Ther 1993;266:820–828.
- Kilpatrick GJ, Dautzenburg FM, Martin GR, et al. 7TM receptors: the splicing on the cake. *Trends Pharmacol Sci* 1999;20: 294–301.
- Rossi GC, Leventhal L, Pan YX, et al. Antisense mapping of MOR-1 in rats: distinguishing between morphine and morphine-6β-glucuronide antinociception. J Pharmacol Exp Ther 1997; 281:109–144.
- Rossi GC, Brown GP, Leventhal L, et al. Novel receptor mechanisms for heroin and morphine-6β-glucuronide analgesia. Neurosci Lett 1996;216:1–4.
- Rossi GC, Pan YX, Brown GP, et al. Antisense mapping of the MOR-1 opioid receptor: evidence for alternative splicing and a novel morphine-6β-glucuronide receptor. FEBS Lett 1995;369: 192–196.
- Pasternak GW, Standifer KM. Mapping opioid receptors using antisense oligodeoxynucleotides: correlating their molecular biology and pharmacology. *Trends Pharmacol Sci* 1995;16:344–350.
- Zimprich A, Simon T, Hollt V. Cloning and expression of an isoform of the rat μ opioid receptor (rMOR1B) which differs in agonist induced desensitization from rMOR1. FEBS Lett 1995; 359:142–146.
- Schuller AGP, King MA, Zhang J, et al. Retention of heroin and morphine-6β-glucuronide analgesia in a new line of mice lacking exon 1 or MOR-1. *Nat Neurosci* 1999;2:151–156.
- Jones KA, Borowsky B, Tamm J, et al. GABA_B receptors function as a heteromeric assembly of the subunits GABA_BR1 and GA-BA_BR1. *Nature* 1998;396:674–679.
- Cejic S, Devi L. Dimerization of the delta opioid receptor: implications for a function in receptor internalization. *J Biol Chem* 1997;272:26959–26964.
- Jordan BA, Devi LA, G-protein coupled receptor heterodimerization modulates receptor function. *Nature* 1999;299:697–700.
- 38. Childers SR. Opioid receptor-coupled second messenger systems. *Life Sci* 1991;48:1991–2003.
- Kieffer BL. Recent advances in molecular recognition and signal transduction of active peptides: receptors for opioid peptides. *Cell Mol Neurobiol* 1995;15:615–634.
- Akil H, Meng F, Devine DP, Watson SJ, Molecular and neuroanatomical properties of the endogenous opioid system: implications for the treatment of opiate addiction. *Semin Neurosci* 1997; 9:70–83.
- 41. Nestler EJ, Erdos JJ, Terwilliger RZ, et al. Regulation by chronic morphine in the rat locus coeruleus. *Brain Res* 1989;476: 230–239.
- Duman RS, Tallman JF, Nestler EJ. Acute and chronic opiate regulation of adenylate cyclase in brain: specific effects in locus coeruleus. J Pharmacol Exp Ther 1988;246:1033–1039.
- Nestler EJ, Tallman JF. Chronic morphine treatment increases cyclic AMP-dependent protein kinase activity in rat locus coeruleus. Mol Pharmacol 1988;33:127–132.
- 44. Nestler EJ. Molecular mechanisms of drug addiction. *J Neurosci* 1992;12:2439–2450.
- Nestler EJ. Drug addiction: a model for the molecular basis of neural plasticity. *Neuron* 1993;11:995–1006.
- Maldonado R, Valverde O, Garbay C, et al. Protein kinases in the locus coeruleus and periaqueductal gray matter are involved

- in the expression of opiate withdrawal. *Naunyn Schmiedebergs Arch Pharmacol* 1995;352:565–575.
- 47. Punch LJ, Self DW, Nestler EJ, et al. Opposite modulation of opiate withdrawal behaviors on microinfusion of a protein kinase A inhibitor versus activator into the locus coeruleus or periaqueductal gray. *J Neurosci* 1997;17:8520–8527.
- 48. Terwilliger RZ, Beitner-Johnson D, Sevarino KA, et al. A general role for adaptations in G-proteins and the cyclic AMP system in mediating the chronic actions of morphine and cocaine on neuronal function. *Brain Res* 1991;548:100–110.
- Self DW, Genova LM, Hope BT, Barnhart W, et al. Involvement of cAMP-dependent protein kinase in the nucleus accumbens in cocaine self-administration and relapse of cocaine-seeking behavior. *J Neurosci* 1998;18:1848–1859.
- Mestek A, Hurley JH, Bye LS, et al. The human mu opioid receptor: modulation of functional desensitization by calcium calmodulin-dependent protein kinase and protein kinase C. Can J Neurosci 1995;15:2396–2406.
- Narita M, Narita M, Mizoguchi H, et al. Inhibition of protein kinase C, but not of protein kinase A, blocks the development of acute antinociceptive tolerance to intrathecally administered mu-opioid receptor agonist in the mouse. *Eur J Pharmacol* 1995; 280:R1–R3.
- Ueda H, Miyame T, Hayashi C, et al. Protein kinase C involvement in homologous desensitization of delta-opioid coupled to G_{i1}-phospholipase C activation in *Xenopus* oocytes. *J Neurosci* 1995;15:78485–78499.
- Keith DE, Murray SR, Zaki PA, et al. Morphine activates opioid receptors without causing their rapid internalization. *J Biol Chem* 1996;271:19021–19024.
- 54. Keith DE, Anton B, Murray SR, et al. Mu-opioid receptor internalization: opiate drugs have differential effects on a conserved endocytic mechanism in vitro and in the mammalian brain. Mol Pharmacol 1998;53:377–384.
- Whistler J, Chuang H, Chu P, et al. Functional dissociation of μ opioid receptor signaling and endocytosis: implications for the biology of opiate tolerance and addiction. *Neuron* 1999;23: 737–746.
- Bohn LM, Lefkowitz RJ, Gainetdinov RR, et al.. Enhanced morphine analgesia in mice lacking beta-arrestin 2. Science 1999;286: 2495–2498.
- Whistler JL, von Zastrow M. Morphine activated opioid receptors elude desensitization by beta-arrestin. *Proc Natl Acad Sci U S A* 1998;95:9914–9919.
- Sora I, Takahashi N, Funada M, et al. Opiate receptor knockout mice define μ receptor roles in endogenous nociceptive responses and morphine-induced analgesia. *Proc Natl Acad Sci U S A* 1997; 94:1544–1549.
- Tian M, Broxmeyer HE, Fan Y, et al. Altered hematopoiesis, behavior, and sexual function in μ opioid receptor-deficient mice. *J Exp Med* 1997;185:1517–1522.
- Matthes HWD, Maldonado R, Simonin F, et al. Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the μ-opioid receptor gene. *Nature* 1996;383: 819–823
- Gaveriaux-Ruff C, Matthes HWD, Peluso J, et al. Abolition of morphine-immunosuppression in mice lacking the μ-opioid receptor gene. *Proc Natl Acad Sci U S A* 1998;95:6236–6330.
- Kitchen I, Slowe SJ, Matthes HW, et al. Quantitative audioradiographic mapping of mu-, delta-, and kappa-opioid receptors in mice lacking the mu-opioid receptor gene. *Brain Res* 1997;778: 73–88.
- 63. Matthes HWD, Smadja C, Valverde O, et al. Activity of the δopioid receptor is partially reduced, whereas activity of the κopioid receptor is maintained in mice lacking the μ-receptor. J Neurosci 1998;18:7285–7295.

- 64. Fuchs PN, Roza C, Sora I, et al. Characterization of mechanical withdrawal responses and the effect of μ-, δ-, and κ-opioid agonists in normal and μ-opioid receptor knockout mice. *Brain Res* 1999;821:480–486.
- 65. Zhu Y, King MA, Alwin GP, et al. Retention of supraspinal delta-like analgesia and loss of morphine tolerance in δ opioid receptor knockout mice. *Neuron* 1999;24:243–252.
- 66. Simonin F, Valverde O, Smadja C, et al. Disruption of the κ-opioid receptor gene in mice enhances sensitivity to chemical visceral pain, impairs the pharmacological actions of the selective κ-agonist U-50,488H and attenuates morphine withdrawal. *EMBO J* 1998;17:886–897.
- 67. Pasternak KR, Rossi GC, Zuckerman A, et al. Antisense mapping KOR-1: evidence for multiple kappa analgesic mechanisms. *Brain Res* 1999;826:289–292.
- Slowe SJ, Simonin F, Kieffer B, et al. Quantitative autoradiography of mu, delta, and kappa1 opioid receptors in kappa-opioid receptor deficient mice. *Brain Res* 1999;818:335–345.
- McGinty JF, Wang JQ. Drugs of abuse and striatal gene expression. Adv Pharmacol 1998;42:1017–1019.
- Konig M, Zimmer AM, Steiner H, et al. Pain responses, anxiety and aggression in mice deficient in pre-proenkephalin. *Science* 1996;383:535–538.
- 71. Brady LS, Herkenham M, Rothman B, et al. Region-specific upregulation of opioid receptor binding in enkephalin knockout mice. *Mol Brain Res* 1999;68:193–197.
- 72. Nishi M, Houtani T, Noda Y, et al. Unrestrained nociceptive response and dysregulation of hearing ability in mice lacking the nociceptin/orphanin FQ receptor. *EMBO J* 1997;16: 1858–1864.
- 73. Koster A, Montkowski A, Schulz A, et al. Targeted disruption of the orphanin FQ/nociceptin gene increases stress susceptibility and impairs stress adaptation in mice. *Proc Natl Acad Sci U S A* 1998;96:10444–10449.
- Devine DP, Watson SJ, Akil H. Nociceptin/orphanin FQ regulates neuroendocrine function of the limbic-hypothalamic-pituitary-adrenal axis. *Neuroscience* 2001;102:541–553.
- 75. Manabe T, Noda Y, Mamimya T, et al. Facilitation of long-term potentiation and memory in mice lacking nociceptin receptors. *Nature* 1998;394:577–581.
- Yaksh TL, Yeung JC, Rudy TA. Systematic examination in the rat of brain sites sensitive to the direct application of morphine: observation of differential effect within the periaqueductal gray. *Brain Res* 1976;114:83–103.
- 77. Akil H, Mayer DJ, Liebeskind JC, et al. Comparison chez le rat entre l'analgésie induite par stimulation de la substance grise periaqueductale et l'analgésie morphinique. *C. R. Acadéemie de Science Paris* 1974;274:3603–3605.
- 78. Akil H, Mayer DJ, Liebeskind JC. Antagonism of stimulation-produced analgesia by naloxone, a narcotic antagonist. *Science* 1976;191:961–962.
- Fields HL, Heinricher MM, Mason P. Neurotransmitters in nociceptive modulatory circuitries. *Annu Rev Neurosci* 1991;14: 219–245.
- 80. Harris JA. Descending antinociceptive mechanisms in the brainstem: their role in an animal's defensive system. *J Physiol Paris* 1996;20:15–25.
- 81. Mansour A, Fox CA, Akil H, et al. Opioid-receptor mRNA expression in the rat CNS: anatomical and functional implications. *Trends Neurosci* 1995;18:22–29.
- 82. Gutstein HB, Mansour A, Watson SJ, et al. Mu and kappa receptors in periaqueductal gray and rostral ventromedial medulla. *Neuroreport* 1998;9:1777–1781.
- Pan ZZ, Tershner SA, Fields HL. Cellular mechanisms for antianalgesic action of agonists of the κ-opioid receptor. *Nature* 1997; 389:382–384.

- 84. Jessel T, Iversen L, Opiate analgesics inhibit substance P release from the rat trigeminal nucleus. *Nature* 1977;268:549–551.
- 85. Yaksh T, Jessell T, Gamse R, et al. Intrathecal morphine inhibits substance P release from mammalian spinal cord *in vivo. Nature* 1980;286:155–157.
- 86. Trafton JA, Abbadie XC, Marchand S, et al. Spinal opioid analgesia: how critical is the role substance P signaling? *J Neurosci* 1999; 19:9642–9653.
- 87. Matthes BK, Franklin K. Formalin pain is expressed in decerebrate rats but is not attenuated by morphine. *Pain* 1992;51: 199–206.
- 88. Manning BH, Morgan MJ, Franklin K. Morphine analgesia in the formalin test: evidence for forebrain and midbrain sites of action. *Neuroscience* 1994;63:289–294.
- 89. Manning BH, Mayer DJ. The central nucleus of the amygdala contributes to the production of morphine antinociception in the rat tailflick test. *J Neurosci* 1995;15:8199–8213.
- 90. Manning BH, Mayer DJ. The central nucleus of the amygdala contributes to the production of morphine analgesia in the formalin test. *Pain* 1995;63:141–152.
- Neal CR, Mansour A, Reinscheid R, et al. Opioid receptor-like distribution in the rat central nervous system: Comparison of ORL1 receptor mRNA expression with ¹²⁵I-(¹⁴Tyr)-orphanin FQ binding. *J Comp Neurol* 1999;412:563–605.

- Neal CR, Mansour A, Reinscheid R, et al. Localization of orphanin FQ (nociceptin) peptide and messenger RNA in the central nervous system of the rat. *J Comp Neurol* 1999;406: 503–547.
- Yammamoto T, Nozaki-Taguchi N, Kimura S. Analgesic effect of intrathecally administered nociceptin, an opioid-receptor like agonist, in the rat formalin test. *Neuroscience* 1997;81:249–254.
- 94. Xu XJ, Hao JX, Wiesendelf-Hallin Z. Nociceptin or antinociceptin: potent antinociceptive effect of orphanin FQ/nociceptin in the rat. *Neuroreport* 1996;7:2092–2094.
- Rossi GC, Leventhal L, Pasternak GW. Naloxone-sensitive orphanin FQ-induced analgesia in mice. Eur J Pharmacol 1996; 311:R7–R8.
- Rossi, GC, Leventhal L, Bolan E. Pharmacological characterization of orphanin FQ/nociceptin and its fragments. *J Pharmacol Exp Ther* 1997;282:858–865.
- 97. Grisel JE, Mogill JS, Belknap JK, et al. Orphanin FQ acts as at supraspinal, but not spinal, antiopioid peptide. *Neuroreport* 1996; 7:2125–2129.
- 98. Heinricher MM, McGaraughty S, Grandy DK. Circuitry underlying antiopioid actions of orphanin FQ in the rostral ventromedial medulla. *J Neurophysiol* 1997;78:3351–3358.
- Zadina JE, Hackler L, Ge LJ, et al. A potent and selective endogenous agonist for the mu-opiate receptor. *Nature* 1997;386: 499–502.