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The Role of Orphanin FQ in the Development of Morphine Tolerance and Dependence

by

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This thesis is dedicated to my lovely wife Diane C. Darland, to my mother Marjorie K. Darland, and to the members of my extended family

"That which does not kill us makes us stronger"

-Friedrich W. Nietzche

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Abstract

The development of tolerance and dependence after chronic exposure to morphine involves at least two processes: diminished efficacy of opioid receptors, and the upregulation of so-called anti-opioid systems which normally homeostatically balance the endogenous opioid system. Several peptides have been classified as having anti-opioid properties including the recently characterized heptadecapeptide orphanin FQ (OFQ). Though similar structurally and functionally at the cellular level to the classical opioid peptides, OFQ displays potent anti-opioid activity with respect to the supraspinally mediated aspects of nociception. OFQ's attenuating effects on analgesia produced by morphine suggested the possibility that the peptide may play a role in the development of tolerance and dependence. This hypothesis is consistent with our observations that mRNA for the OFQ precursor, preproOFQ (ppOFQ) and receptor (OFQR), as detected by in situ hybridization, is expressed in several brain regions that are believed to be important in the development of morphine dependence.

Therefore we tested our hypothesis by evaluating changes in OFQ peptide, ppOFQ mRNA and OFQR mRNA in mice rendered morphine tolerant and dependent. We found significant increases in OFQ peptide immunoreactivity in the anterior hypothalamus, ventral midbrain, dorsal pons and ventral medulla of morphine-dependent mice. This was accompanied by an increase in ppOFQ mRNA in the ventral forebrain and a decrease in the amygdala, as measured by RNase protection assays. In situ hybridization was used to expand the ppOFQ mRNA findings. Using in situ hybridization we detected a modest increase of ppOFQ mRNA in the ventral medulla and a decrease in the medial amygdala in the brains of morphine-dependent mice.

Next we tested the hypothesis that OFQ contributes to morphine dependence by administering an intracerebroventricular (icv) injection of OFQ peptide in to dependent mice and evaluating the precipitation of withdrawal symptoms. OFQ precipitated mild withdrawal symptoms in both morphine-dependent and naive mice. In addition, the

peptide decreased total locomotion in both morphine-dependent and naive animals and decreased rearing specifically in dependent animals.

The changes in the OFQ system after chronic morphine exposure are consistent with the idea that the peptide is involved in the development of morphine tolerance and dependence. The failure of OFQ to precipitate many of the classic opiate withdrawal signs in dependent mice suggests that the peptide may not be involved in the development of physical dependence. However, due to current experimental limitations, the possibility cannot be completely dismissed. The decreases in locomotion and rearing observed in morphine-dependent animals treated with OFQ suggest the possibility that the peptide may be involved in the development of certain affective aspects of dependence.

Introduction

1. Opiate Receptors and Opioid Peptides: An Historical Perspective

Opiates are alkaloid compounds derived from the poppy plant, *Papaver somniferum*. They have been used medicinally and recreationally for centuries because of their ability to produce analgesia, alter mood and control diarrhea. Equally long is the history of opiate addiction, a pattern of behavior characterized by an obsessive preoccupation with the procurement and use of the drug as well as a frequent return to use after periods of abstinence. One major factor contributing to the phenomenon of opiate addiction is the reinforcing property of these compounds. That is, use of an opiate drug modifies the behavior of the individual such that there is a higher likelihood of the drug being used again. Also, contributing to opiate addiction are tolerance, a term which refers to the diminished effectiveness of the drug after repeated exposure, and physical dependence, a physiological state that can be reached in which abstinence from the drug or blockage of the drug's effects by a pharmacological antagonist results in a characteristic withdrawal syndrome (Jaffe, 1990). The tendency for humans to take opiates and other drugs of abuse is shared by other mammals. Rats and mice for example will self-administer opiates, display tolerance as well as dependence and can thus serve as models to study various aspects of opiate addiction.

Our understanding of the molecular processes underlying the actions of opiates took a great leap forward in the early 1970's with the discovery of specific, high affinity opiate binding sites in brain and certain peripheral tissues including the guinea pig ileum and the mouse vas deferens (Pert & Snyder, 1973; Simon *et al.*, 1973; Terenius, 1973). It was soon recognized that there was more than one form of binding site, since different opiate compounds exerted distinct effects on analgesia and display unique dose response curves. Observations such as these led Gilbert and Martin to postulate the existence of a mu (μ) receptor which bound morphine, a kappa (κ) receptor which bound ketocyclazocine, and a

sigma (σ) receptor which bound allylnorcycazocine (SKF-10,047) (Gilbert & Martin, 1976). Comparing the binding profiles of opiates, as well as certain endogenous opioid peptides (see below) in brain, ileum and vas deferens, it was concluded that the three tissues expressed distinct classes of receptors (Hutchinson *et al.*, 1975; Lord *et al.*, 1977). The vas deferens displayed a unique binding profile which led to the naming of a new type of opiate receptor, the delta (δ) receptor. Thus, pharmacological profiles of various opiate agonists and antagonists demonstrated four major classes of binding sites μ, δ, κ and σ. Subsequent studies showing that the effects modulated by the σ receptor were neither stereoselective nor reversible by naloxone have been cited as evidence that the σ receptor is not a classic opiate receptor (Quirion *et al.*, 1987).

The discovery of discrete, high affinity and stereospecific opiate binding sites eventually led to the characterization of a family of endogenous opiate receptor ligands known as opioid peptides. Before these binding sites were actually demonstrated experimentally it was believed that opiate drugs must exert their effects through endogenous receptors whose normal function *in vivo* must involve endogenous neuromodulatory molecules (Collier, 1972). Therefore, after demonstrating the existence of the receptors an intensive effort began to identify their endogenous ligands.

Initial successes in this regard relied on use of the guinea pig ileum (GPI) and mouse vas deferens (MVD) as bioassay systems that could be used to assess opioid activity in extracts prepared from whole brains and isolated pituitary glands of various species (Hughes *et al.*, 1975a; Pasternak & Snyder, 1975a; Terenius, 1975a). Others developed opiate binding assays in whole brain to demonstrate endogenous opioids (Terenius & Wahlstrom, 1975b).

The first opioid peptides to be identified were met- and leu-enkephalin (Hughes *et al.*, 1975b), two pentapeptides that differ only in their C-terminal residue. Both of these peptides are derived from a larger hormone precursor that would later be named preproenkephalin (Noda *et al.*, 1982). The next endogenous opioids to be discovered were the ß-endophin peptides derived from the C-terminus of what later came to be called proopiomelanocortin or POMC (Bradbury *et al.*, 1977; Li & Chung, 1976). The last classic opioid peptide discovered was dynorphin which was purified from porcine pituitary gland extracts (Goldstein *et al.*, 1981). It too was found to be derived from a larger protein precursor called preprodynorphin (Kakidani *et al.*, 1982).

By examining the binding profiles of several synthetic and endogenous ligands, each of the opiate receptor types have been assigned an endogenous peptide (Goldstein & Naidu, 1989). In this scheme the enkephalins are believed to be the endogenous ligands for the delta opioid receptor (DOR) and the dynorphins are believed to be the endogenous ligands for the kappa opioid receptor (KOR) (Chavkin *et al.*, 1982). The endogenous ligands for the mu opioid receptor (MOR), however has proven more elusive. ß-endorphin was initially considered to be the endogenous ligand because of its high affinity for MOR. However, in spite of its high binding affinity, ß-endorphin lacks specificity as evidenced by its high affinity for DOR. Recently, two tetrapeptides, the endomorphins, were purified and shown to bind MOR with high specificity and high affinity (Zadina *et al.*, 1997). Presently these peptides are considered promising candidates to be the endogenous ligands for MOR.

Due to the considerable overlap in the affinities of various ligands for the three main types of opioid receptors there has been an ongoing search for more specific agonists and antagonists. This search has yielded considerable pharmacological evidence for the existence of multiple subtypes of opiate receptor family members including two MOR (μ1

and μ2) (Pasternak & Wood, 1986), two DOR (δ1 and δ2) (Mattia *et al.*, 1991; Sofuoglu *et al.*, 1992), as well as three KOR subtypes (κ1, κ2 and κ3) (Clark *et al.*, 1989).

Despite the wealth of knowledge gathered regarding the properties of the various opioid receptor subtypes, it was not until 1992 that the first opiate receptor, DOR, was actually cloned (Evans *et al.*, 1992; Kieffer *et al.*, 1992). This was followed the next year by the cloning of MOR and KOR by several groups (Bunzow *et al.*, 1995; Chen *et al.*, 1993a; Chen *et al.*, 1993b; Li *et al.*, 1993; Meng *et al.*, 1993; Minami *et al.*, 1993; Thompson *et al.*, 1993; Yasuda *et al.*, 1993). A fourth receptor, homologous to the opiate receptors and yet quite unique pharmacologically, was also cloned at this time (Bunzow *et al.*, 1994; Chen *et al.*, 1994; Mollereau *et al.*, 1994; Wang *et al.*, 1994). The nucleotide sequence of these receptors has revealed that all four are members of the seven transmembrane domain G-coupled receptor super family (the alignment and structure of G-coupled receptors is reviewed by Probst *et al.*, 1992). The overall amino acid homology between the opiate receptor family members is approximately 70%, with the greatest conservation localized to the transmembrane domains and intracellular loops. The extracellular loops and the N-termini are the most divergent regions between family members, reflecting their different binding profiles. Interestingly, the search for additional opioid receptor genes has failed to provide an explanation for the multiple subtypes characterized pharmacologically.

Before the cloning of the opiate receptors in the early 1990's, it had already been established that they were G-coupled receptors . It was first described in NG108 cells and then later confirmed in brain and peripheral tissues that opiate binding is regulated by guanine nucleotides and causes a decrease in adenylyl cyclase (AC) activity (reviewed by Childers, 1991). Blocking the opiate-induced decrease in AC activity with pertussis toxin confirmed that the opiate receptors were coupled to G_i/G_o (Puttfarcken *et al.*, 1988; Yu *et al.*, 1986). The opiate receptors were also found to activate inwardly rectifying K^+

channels either via G_i or G_o, thereby hyperpolarizing neurons and decreasing their excitability (Gross *et al.*, 1990; North *et al.*, 1987). Opiate receptors also inhibit voltage-gated Ca^{2+} channels and thereby decrease synaptic transmission both pre- and post-synaptically (Hescheler *et al.*, 1987; Rhim & Miller, 1994).

A combination of molecular biology, pharmacology and behavioral studies has revealed multiple roles for the endogenous opioid system. Receptor autoradiography and *in situ* hybridization were used to elucidate the sites of expression of the various receptor subtypes (reviewed by Mansour *et al.*, 1995). In many instances the expression patterns corresponded with functional data obtained from behavioral studies. One of the best examples of this correspondence is in the opiate regulation of the mesolimbic dopamine system. Morphine stimulates dopamine release in the nucleus accumbens and stimulates activity of dopaminergic neurons in the ventral tegmental area (VTA) by hyperpolarizing inhibitory interneurons containing γ-aminobutyric acid (GABA) (Johnson & North, 1992; Leone *et al.*, 1991). Dynorphin opposes these effects on dopaminergic neurons through receptors expressed on post-synaptic terminals in the VTA and on pre-synaptic terminals in the nucleus accumbens (DiChiara & Imperato, 1988a; Mansour *et al.*, 1995). This role in regulating mesolimbic dopaminergic neuronal activity and therefore reinforcing behavior is thought to account, in part, for the action of opioids on feeding and sexual behavior (Browne & Segal, 1980) and may also account for some of the abuse potential of opiates (DiChiara & Imperato, 1988b; Wise & Rompre, 1989). Endogenous opioids have been shown to regulate release of hormones from the hypothalamic-pituitary axis, including prolactin, growth hormone, corticotropin releasing factor (CRF), POMC-derived peptides, lutenizing hormone, oxytocin and vasopressin (Carter *et al.*, 1984; Leadem & Yagenova, 1987; Pfeiffer *et al.*, 1987; Slizgi & Ludens, 1982). Opioids are also believed to play a role in memory and learning by their action in the hippocampus (reviewed by Morris & Johnston, 1995). Other functions attributed to the opioids include temperature and

cardiopulmonary regulation (reviewed by Stanley, 1987). However, the most studied action of the endogenous opioid system relates to analgesia,that is, the regulation of pain perception.

Morphine and other opioid ligands produce their analgesic effects by modulating the ascending and descending pain pathways (reviewed by Basbaum & Fields, 1984; Fields *et al.*, 1991). Nociceptive information, including pain, is relayed from sensory neurons in the periphery to neurons in the superficial dorsal horn of the spinal cord. These spinal neurons project via the spinothalamic and spinorecticular tracts to the ventral posterior thalamus and the reticular formation in the medulla respectively. Input coming from the periphery is also modulated by a descending pathway which has major relay points in the periaqueductal gray (PAG) and the raphe magnus nucleus (RM) of the rostral ventral medulla (RVM). All three opioid receptor subtypes are expressed spinally and in dorsal root ganglia (DRG) which contain the cell bodies of the peripheral sensory neurons (Mansour *et al.*, 1995). Supraspinally, MOR and KOR are the primary receptor types in the thalamus, PAG, and RM. Morphine can therefore exert analgesic effects, that is block painful stimuli, by binding to receptors in the spinal cord and directly inhibiting transmission from the periphery or by binding receptors and regulating the output of supraspinal modulatory areas such as the PAG and the RVM. The RVM controls spinal transmission by two main classes of neurons which change their firing patterns in response to painful stimuli (Fields *et al.*, 1991; Heinricher *et al.*, 1994). The first class of neuron is called an "on" cell because its activity increases during the occurrence of nociceptive reflexes during the transmission of painful stimuli, the second class is called an "off" cell because its activity is inhibited just prior to the onset of a nociceptive reflex. Off cells are activated by morphine and are therefore thought to be key regulators of supraspinal analgesia. The input to the RVM from the PAG is also regulated by morphine and is thought to involve regulation of the off neurons. Analgesia is the hallmark activity of the

opioid activity and has also provided the most reliable means of measuring the development of tolerance to the effects of morphine.

2. Morphine Tolerance and Physical Dependence

Two of the defining characteristics of morphine's action are the phenomena of tolerance and dependence. Tolerance is a decreased effectiveness of a drug to produce a desired effect after chronic exposure. Dependence is defined as the adaptive state that is reached after chronic exposure such that the continued presence of the drug is required to prevent the onset of withdrawal symptoms. Both opiate tolerance and dependence have been studied in several model systems including cultured cell lines, isolated tissue and in whole animal.

Soon after the discovery of opiate binding sites in neural tissues the neuroblastoma x glioma hybrid cell line NG108-15 was found to densely express opiate receptors, specifically DOR (Sharma *et al.*, 1975a). It was found that the addition of opiate agonists to NG108 cultures blocked forskolin-induced increases in cAMP. However, after chronic treatment with agonist, the opiate-induced inhibition of AC activity decreased (Sharma *et al.*, 1975b). In this paradigm the development of tolerance to morphine occurs in two stages (Lefkowitz *et al.*, 1980; Su *et al.*, 1980). The first stage involves receptor desensitization where the receptor is uncoupled from the G protein signal transduction machinery resulting in both decreased affinity and efficacy. The molecular mechanism by which this occurs is not clearly understood but, it appears to involve the phosphorylation state of the receptor (Louie *et al.*, 1986). The second stage involves receptor downregulation, an actual decrease in receptor number. Upon cessation of morphine treatment after a period of chronic exposure, NG108 cells display elevated cAMP levels. This elevated level of cAMP has been proposed to be a molecular sign of withdrawal. The mechanism for this increase in cAMP from an increased activity of adenylate cyclase may

involve stimulation by Ca^{2+}/calmodulin (Law *et al.*, 1984). Thus, dependence in the NG 108 cell line appears to involve events downstream of the opiate receptor.

Investigators also began to study whole tissue preparations with the hope of understanding the molecular bases of tolerance and dependence. The favorite tissue preparations for these studies have been the guinea pig illeum (GPI) and mouse vas deferens (MVD) because, unlike brain slice preparations, complex neural circuitry is not a concern and because both display well characterized responses to chronic morphine (Kosterlitz & Paterson, 1980; Kosterlitz & Waterfield, 1975). Upon treatment with morphine both smooth muscle preparations display a profound decrease in electrically stimulated contraction. However, this response diminishes over time as tolerance develops to chronic morphine administration. In the GPI, a dependent state can be reached in which naloxone precipitates increased spontaneous contraction.

Two of the most important contributions of the work employing the GPI and MVD concern issues of cross-tolerance and cross-dependence. Unlike NG108 cells these tissues express more than one type of opiate receptor. The GPI expresses MOR and KOR, the MVD expresses predominantly DOR but also low levels of MOR and KOR. It is therefore possible to determine whether chronically activating one subtype creates tolerance and dependence to ligands of the other type. Earlier studies involving the MVD from animals made tolerant to DOR selective agonists demonstrated that little tolerance was developed to MOR or KOR agonists (Schulz *et al.*, 1980). However, more recent work demonstrating, for example, some cross-tolerance between MOR and KOR subtypes in the GPI (Garaulet *et al.*, 1995) indicate that the issue is still open to question. There is some evidence that cross-dependence develops in the GPI, however, these studies were confounded by the fact that a certain degree of dependence was actually lost during the preparation of the tissue

and because naloxone, an antagonist with relatively low specificity, was used to precipitate withdrawal (Schulz *et al.*, 1980).

Though still a controversial issue, the absense of cross-tolerance in the GPI and MVD reflects the importance of the opiate receptor in the development of tolerance. This is in stark contrast to the mechanism involved in the development of dependence in these tissues. Interestingly, a dependent state identical to that induced by chronic morphine can be achieved in the GPI with chronic exposure to adrenergic agonists (Collier & Tucker, 1984). Withdrawal precipitated by antagonists against one receptor type can be blocked by applying agonist to the other receptor type. Thus, dependence, as in the case of the NG108 cell line, involves events downstream of the receptor. In contrast to NG108 cells, however, dependence in the GPI, while G-protein-dependent, does not appear to be due to increased AC activity (Collier & Tucker, 1984; Lux & Schulz, 1983). Thus, the situation in the GPI is more complicated than NG108 cells, as might be expected in a multicellular system. More complicated still is the situation in brain tissue where it was discovered early on that intact neural circuitry is crucial for the development of tolerance and dependence (Andrade *et al.*, 1983; North & Williams, 1983). Consequently, whole animals continue to be an important focus of research efforts concerning tolerance and dependence.

As in humans, naloxone administered to morphine-dependent animals can precipitate withdrawal, or an abstinence syndrome which is composed of several well-characterized physical and motivational symptoms. In the case of rats and mice the physical signs of withdrawal include escape jumping, wet dog shakes, writhing, facial grimacing, teeth chattering, pilo erection, ptosis, diarrhea and hypothermia (Blasig *et al.*, 1973; Way *et al.*, 1973). Motivational symptoms have proven more difficult to quantify though attempts have been made to measure aversion to locations where abstinence occurred (Hand *et al.*, 1988; Manning & M.C. Jackson, 1977; Mucha, 1987); disruption of trained operant

behavior such as lever pressing for food (Gellert & Sparber, 1977); as well as free range locomotor activity (Higgins & Sellers, 1994; Schulteis *et al.*, 1994). For both the physical and motivational symptoms of withdrawal, multiple brain regions have been implicated as relevant substrates.

The stereotaxic injection of opiate antagonists into the brains of dependent animals, followed by the scoring of withdrawal signs, has been used to map the regions involved (Maldonado *et al.*, 1992). Of the areas tested, the locus coeruleus (LC) showed the greatest global withdrawal score although areas such as the PAG, RM and hypothalamus had high scores as well at the same dosage. At higher concentrations of antagonist the nucleus accumbens, medial amygdala and medial thalamus also appeared to mediate withdrawal. Other physical symptoms such as diarrhea, salivation, lacrimation or rhinorrhea were not precipitated by intracerebroventicular (icv) injection or stereotaxically delivered antagonist suggesting that these symptoms were mediated by peripheral opiate receptors. This hypothesis has been supported by work with peripherally acting antagonists in opiate-dependent animals (Bianchetti *et al.*, 1986). Motivational signs of withdrawal also appeared to be localized to multiple sites in the brain with the best characterized areas being the nucleus accumbens and amygdala (Koob *et al.*, 1989; Stinus *et al.*, 1990).

Experiments designed to explore the mechanisms underlying the cellular basis for tolerance and dependence *in vivo* have built upon the earlier work involving NG108 cells and have primarily focused on the LC (reviewed by Nestler, 1994; Nestler & Aghajanian, 1997). As in the neuroblastoma cell line, morphine inhibits AC and cAMP production in neurons of the LC. In the LC of morphine-dependent animals the cAMP signalling system is upregulated in response to the chronic inhibition imposed by chronic morphine. Blocking the effects of morphine then leads to higher levels of cAMP. The elevation of

cAMP in LC neurons is thought to constitute withdrawal at the cellular level. In the LC upregulation of the cAMP system is responsible for the increased electrical output of the nucleus, which has been correlated with the onset of withdrawal symptoms. Additional evidence that the cAMP pathway is important in the development of dependence comes from studies which show attenuated withdrawal severity in mutant mice deficient for CREB (Maldonado *et al.*, 1996). There is, however, still some debate as to whether the LC is as central in precipitating opiate withdrawal as earlier studies had suggested (Chieng & Christie, 1995; Christie *et al.*, 1997).

While the effects of chronic morphine exposure on the MOR and downstream effectors are undoubtedly important for the development of tolerance and dependence, there is considerable evidence that additional factors are also involved. For example, it has been estimated that the degree of tolerance achieved *in vitro* is far less than what is achievable *in vivo* (reviewed in (Smith *et al.*, 1989). Despite several efforts, whether a decrease in opiate receptor density occurs in tolerant animals remains a controversial issue with some groups reporting mild upregulation of binding sites and receptor mRNA (Brady *et al.*, 1989; Lewis *et al.*, 1984; Rothman *et al.*, 1989); downregulation (Bhargava & Gulati, 1990; Diaz *et al.*, 1995; Nishino *et al.*, 1990; Ronnekleiv *et al.*, 1996; Tao *et al.*, 1987; Tempel *et al.*, 1988); or no change at all (Dum *et al.*, 1979; Hitzemann *et al.*, 1974) depending on the efficacy of the agonist used and the duration of the treatment. To date the changes that have been reported are too small to account for the degree of tolerance achieved *in vivo*. Also, in contrast to what is observed in NG108 cells, the amount of naloxone required to precipitate withdrawal *in vivo* decreases with increased tolerance (Wei *et al.*, 1973). Finally, in brain slices it has been possible to demonstate that in many instances external circuitry is required for the electrophysiological changes reported during the development of tolerance and dependence *in vivo* (Andrade *et al.*, 1983; North & Williams, 1983). Therefore, in addition to the role played by the endogenous opioid

system, other neurochemical systems in the brain are thought to be required for the development of tolerance and dependence *in vivo*.

3. Anti-opioid Peptides

A growing literature implicates other neuromodulators, particularly certain peptides, in the development of opiate tolerance and dependence (Rothman, 1992). It has been proposed that these "anti-opioid" peptides normally function to balance the opioid system and are therefore upregulated in response to chronic activation of the opioid receptors. This upregulation of the opposing anti-opioid system contributes to tolerance development. Finally, in the dependent state, cessation or blocking the endogenous opioid system creates an abnormal predominance of anti-opioid activity which manifests itself in various withdrawal symptoms.

Several neuromodulatory systems have been characterized as having anti-opioid function. N-methyl-D-aspartate (NMDA) antagonists as well as nitric oxide synthase inhibitors both inhibit the formation of tolerance and dependence suggesting a role for glutamatergic transmission (Higgins *et al.*, 1992; Kolesnikov *et al.*, 1993; Trujillo & Akil, 1991). In addition, the actions of several peptides normally antagonistic to the effects of morphine have been implicated as potentially important contributors to tolerance and dependence including dynorphin (DYN), itself an opioid, cholecystokinin (CCK), thyrotropin releasing hormone (TRH), α-melanocyte stimulating hormone (α-MSH) and neuropeptide FF (NPFF).

DYN A, an endogenous ligand for the KOR, has been shown to antagonize the effects of MOR agonists on spinal-mediated analgesia via descending, anti-analgesic projections (Fujimoto *et al.*, 1990a). In addition, this pathway contributes to the tolerance developed to a single large dose of morphine (Fujimoto & Holmes, 1990b). Finally, while not

necesarily connected to tolerance or dependence, KOR agonists have been shown to produce dysphoria and oppose the rewarding effects of morphine in rodents and humans (DiChiara & Imperato, 1988a; Pfeiffer *et al.*, 1986). Consequently, these anti-opiate effects may contribute to repeated drug self-administration .

CCK, another putative anti-opioid peptide, has been demonstrated to have a biphasic activity with respect to pain thresholdwhen administered intraperitoneally (ip): high doses induce analgesia whereas low, physiological doses oppose morphine-induced analgesia (Faris *et al.*, 1983; Zetler, 1980). Furthermore, animals rendered analgesic by chronic footshock displayed less analgesia when given either systemic or intrathecal CCK. Also, animals immunized against CCK displayed heightened analgesia (Faris, 1985). Finally, CCK receptor antagonists can enhance morphine analgesia and prevent the development of morphine tolerance (Dourish *et al.*, 1990).

NPFF was initially indentified in bovine brain extracts using an antisera that had been developed against a molluscan peptide, FMRF-NH2, a peptide that has cardiovascular effects in mammals (Yang *et al.*, 1985). When administered ip, NPFF dose-dependently attenuates the antinociceptive activity of morphine, as well as certain opioid peptides, and delays the development of tolerance in rats (Tang *et al.*, 1984a). Subsequently, it was found that NPFF injected icv and NPFF analogs administered systemically could precipitate withdrawal symptoms in morphine-dependent rats (Malin *et al.*, 1990b; Malin *et al.*, 1995). Furthermore, antisera to the peptide, as well as synthetic analogs, prevented naloxone-induced withdrawal symptoms (Lake *et al.*, 1991; Malin *et al.*, 1993a). When NPFF was injected icv the authors also reported that the peptide precipitated withdrawal signs in drug naive animals, inducing a "quasi-abstinence" state which was reversible by morphine (Malin *et al.*, 1990b). NPFF immunoreactivity is also significantly upregulated in various brain regions and in the spinal cord during chronic morphine exposure (Tang *et*

al., 1984a). Taken together these data suggest a prominant role for the peptide in opiate tolerance and dependence.

4. OFQ and its Receptor

In addition to the action of these peptides a new neuropeptide has been isolated which also has anti-opioid properties. In 1994 four groups simultaneously reported the cloning of an additional member of the opioid receptor gene family (Bunzow *et al.*, 1994; Chen *et al.*, 1994; Mollereau *et al.*, 1994; Wang *et al.*, 1994). One group called the receptor LC132 because it was cloned from a rat locus coeruleus cDNA library (Bunzow *et al.*, 1994), while another group called it opioid receptor-like receptor, or ORL1, because of its unique pharmacology (Mollereau *et al.*, 1994). The deduced amino acid sequence of this receptor shared 65% identity overall with the other opioid receptors and was particularly similar in the seven putative transmembrane domains and the second and third intracellular loops. It was, therefore, predicted that this newly discovered receptor would resemble the opiate receptors in that it would couple to G_i/G_o proteins and affect the same second messenger systems including AC, K^+ and Ca^{++} channels. Interestingly the new receptor was quite divergent from the classic opiate receptors in its second and third extracellular loops, as well as the N-terminal. All these regions are believed to be important for ligand binding. Indeed, pharmacological studies on transiently and stably transfected cells failed to show appreciable binding to the classic opioid ligands or peptides. The pronounced lack of opiate or opioid binding stimulated the search for the endogenous ligand of this orphan receptor.

In 1995 two groups simultaneously reported the isolation and characterization of an endogenous ligand for the orphan opiate receptor LC132/ORL1 (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995). The peptide consists of 17 amino acid residues and is very similar in sequence and charge density to dynorphin. One group named the peptide

orphanin FQ (OFQ) because it is a ligand for the orphan receptor and because of its N-terminal phenylalanine (F) and C-terminal glutamine (Q) residues (Reinscheid *et al.*, 1995). The other group named the peptide nociceptin for its apparent pronociceptive activity in certain analgesic assays (Meunier *et al.*, 1995). Distinct from dynorphin and the other classic opioid peptides are the N-terminal and middle residues (see Figure 1). The N-terminal can be changed to a tyrosine, thereby rendering OFQ more opioid-like without affecting its binding or activity. The middle residues, however, give OFQ its very distinct binding characteristics. OFQ is also similar to the classic endogenous opioid peptides in that it is synthesized as a larger precurser, called preproOFQ (ppOFQ), which is proteolytically processed yielding the mature peptide as well as additional peptides that may have bioactivity (Mollereau *et al.*, 1996; Nothacker *et al.*, 1996).

OFQ induces cellular effects similar to those reported for the classic opioid peptides. In cell lines expressing the OFQ receptor (OFQR), OFQ blocks forskolin-stimulated cAMP accumulation (Reinscheid *et al.*, 1995). Therefore, OFQR behaves as the classic opiate receptors by coupling negatively to AC via G_i/G_o. Furthermore OFQR has been shown to couple to an inwardly rectifying K^+ current when expressed in Xenopus oocytes (Ikeda *et al.*, 1997) as well as neurons of the locus coeruleus (Conner *et al.*, 1996); the dorsal raphe (Vaughn & Christie, 1996); the periaqueductal gray (Vaughn *et al.*, 1997) and the arcuate nucleus of the hypothalamus (Wagner *et al.*, 1998). Thus, OFQR behaves as the other opioid receptors in that one of its functions is to hyperpolarize neurons. Again as with its other family members OFQR couples negatively to N, L, P and Q type voltage-gated Ca^{2+} channels (Connor *et al.*, 1996; Knoflach *et al.*, 1996). The OFQ system thereby inhibits synaptic transmission as demonstrated in the dorsal horn of the spinal cord, the

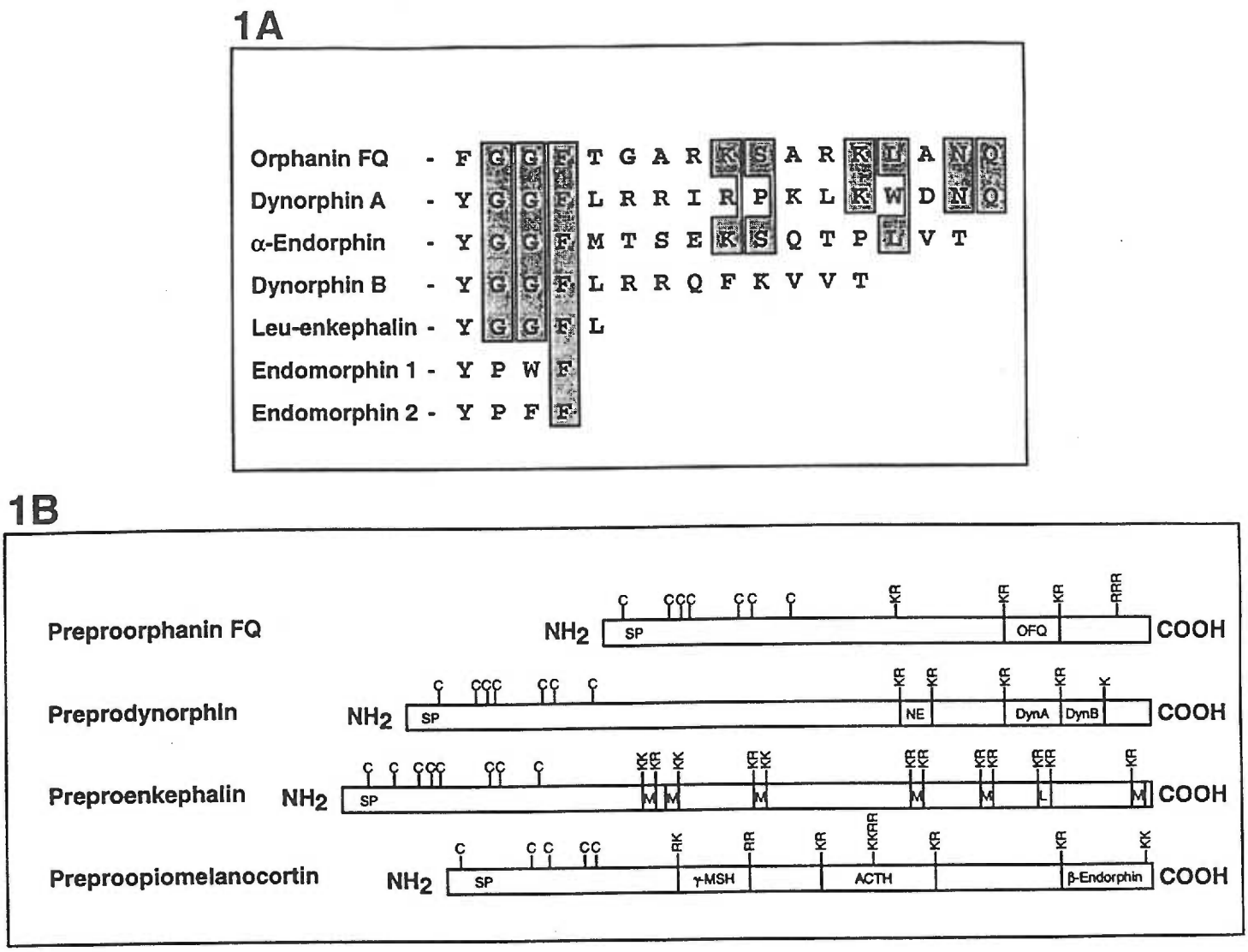


Figure1. Comparison of the OFQ peptide sequence and precursor structure with the other opioids. (A) Alignment of the OFQ peptide sequence with the other opioid peptides. Shaded areas represent homologous residues between OFQ and the other peptides (adapted from Reinscheid *et al.*, 1995). Endomorphin sequences are from Zadina *et al.*, 1997. (B) Comparison of ppOFQ with the precursors of the other opioids. The positions of the cysteine (C) residues and basic amino acids (K and R) are shown. The major peptide products of the precursor downstream from the signal peptide (SP) are shown as shaded boxes including α-neoendorphin (NE), dynorphin A (DynA), dynorphin B (DynB), Leu-enkephalin (L), Met-enkephalin (M) and γ-melanocyte stimulating hormone (γ-MSH), adrenocorticotrophic hormone (adapted from Mollereau *et al.*, 1996).

hippocampus and the cerebral cortex (Faber *et al.*, 1996; Knoflach *et al.*, 1996; Liebel *et al.*, 1997; Nicol *et al.*, 1996; Wang *et al.*, 1996).

Several functions for the OFQ system have been hypothesized based on its anatomical distribution. Most of these will be discussed more extensively in Chapter 1. For the purposes of introduction however, it is important to describe OFQ's role in pain and analgesia. Initially it was assumed that because of the similarity to other opioid peptides, OFQ would induce analgesia, however, the story has become more complicated and somewhat controversal. Early reports that described supraspinal hyperalgesia failed to account for stress-induced analgesia (SIA) resulting from the icv procedure which raised baseline values (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995). Later it was revealed that OFQ can produce hyperalgesia but actually opposes an apparent opioid-mediated SIA (Mogil *et al.*, 1996a). In addition, it was found that icv administered OFQ also opposed the analgesic effects of morphine, endogenous opioid peptides and electroacupuncture (Mogil *et al.*, 1996b; Mogil *et al.*, 1996a; Tian *et al.*, 1997b). Interestingly, at the level of the spinal cord OFQ can have analgesic properties similar to those of other opioid peptides (Grisel *et al.*, 1996; Tian *et al.*, 1997a).

It must be pointed out that the issue of OFQ's analgesic properties is still controversial. Some groups have reported no supraspinal analgesia while one group reported hyperalgesia followed by analgesia (Grisel *et al.*, 1996; Rossi *et al.*, 1997; Rossi *et al.*, 1996; Tian *et al.*, 1997a). Similarly, while I report here that intrathecally administered OFQ produces analgesia, some groups have reported that the peptide has no detectable effect (Erb *et al.*, 1997; Hao *et al.*, 1997; King *et al.*, 1997; Tian *et al.*, 1997a; Xu *et al.*, 1997; Yamamoto *et al.*, 1997). Still other groups maintain that the peptide produces hyperalgesia when injected intrathecally (Hara *et al.*, 1997). Several differences between studies might contribute to the disparate results including the sex, strain and state of the animals in the studies. Also,

OFQ administered intrathecally or icv is likely to simultaneously affect several behaviors which might confound performance in analgesic assays. This said, there is still considerable evidence for the supraspinal anti-opioid activity of OFQ.

Based on anatomical evidence, the two most likely areas for OFQ's action on opioid-mediated, supraspinal analgesia are the PAG and RVM. The OFQR is expressed at moderate to high levels in these areas (see chapter 1). OFQ inhibits neurons in the PAG (Vaughn *et al.*, 1997) and blocks the antinociceptive effects of morphine injected into this area (Morgan *et al.*, 1997). OFQ also inhibits the firing of all neurons tested in the RVM, including those of the RM, and thus blocks actions of morphine in that area as well (Heinricher *et al.*, 1997). The opposing effects of OFQ on morphine induced analgesia in these areas suggests a possible role for the peptide in the development of analgesic tolerance. Both the RVM and the PAG, as well as several other areas rich in OFQ and OFQR expression (see chapter 1), have also been suggested as potential neural substrates for opiate dependence (reviewed by Koob *et al.*, 1992). It has yet to be demonstrated whether OFQ plays a significant role in the formation of opiate tolerance or dependence.

5. Contributions of This Study

To aid in understanding the potential roles of OFQ, I sought to determine the sites of ppOFQ and OFQR expression by performing extensive anatomical surveys in the rat and mouse brain using *in situ* hybridization. Other groups have published partial *in situ* surveys for ppOFQ mRNA (Houtani *et al.*, 1996; Nothacker *et al.*, 1996) and for the receptor (Bunzow *et al.*, 1994). The studies I present in Chapter 1 are in press, and not only verify but also expand these findings (Darland & Grandy, 1998; Darland *et al.*, 1998). These studies also stimulated additional research at Oregon Health Sciences University that

has attempted to elucidate actions of OFQ *in vivo* (C. Allen et al in preparation and Quigley *et al.*, 1998).

If OFQ is indeed a member of the growing list of anti-opioid peptides, its levels would be expected to increase in response to chronic morphine exposure. Upregulation of the peptide is indeed demonstrated in Chapter 2, which is consistent with it playing a role in the development of morphine tolerance. Finally, anti-opioid peptides are believed to mediate some of the classic withdrawal symptoms seen in opiate-dependent animals upon administration of naloxone. In Chapter 2, I describe studies in which I test whether icv injection of OFQ into morphine dependent animals precipitates withdrawal symptoms. While these experiments demonstrated that OFQ does not appear to mediate the physical aspects of opiate withdrawal the data are consistent with the interpretation that the peptide may have affected certain motivational aspects. Together, my results place OFQ among the other anti-opioid peptides as an important modulator of opiate action at several neuroanatomical levels and may prove to be an important therapuetic target for the treatment of opiate addiction.

Materials and Methods

1. Rat cDNA Probes

A nearly full-length rat OFQ cDNA clone was generated using oligonucleotide primers based on the published sequence (Meunier *et al.*, 1995) in an RT-PCR strategy. The resulting PCR product was subjected to automated sequencing analysis to confirm its identity. A 244 base pair PstI fragment containing the derived amino acid sequence for the mature OFQ peptide was subcloned into pBluescript from which the sense (T3 polymerase) and antisense (T7 polymerase) were generated. The 300 base pair rat OFQR cDNA construct used in these studies is the same probe used to characterize the receptor distribution previously (Bunzow *et al.*, 1994).

2. *In Situ* Hybridization

The *in situ* hybridization was performed essentially as described by Simmons *et al.*, 1989. Briefly, adult male Sprauge-Dawley rats approximately 200g and 3.5 months of age (generously provided by the laboratories of Drs. John Williams and Felix Eckenstein) were anesthetized with isoflurane and perfused with 4% paraformaldehyde dissolved in borate, pH 9.5. Brains and spinal cord were dissected and infiltrated overnight in the same fixative plus 20% sucrose. The tissue was then embedded in OCT compound and sectioned on a cryostat. Floating sections 25 µm thick were mounted onto Superfrost Plus slides (VWR). The slides were then fixed in 4% paraformaldehyde dissolved in PBS; permeabilized with proteinase K; acetylated in acetic anhydride and triethanolamine and dehydrated in ethanol.

Radioactive riboprobes were synthesized in the presence of ^{35}S UTP with the appropriate polymerase (see above). Riboprobes were purified on Sephadex G-50 columns (Pharmacia) and then diluted to a final concentration of 2.5 $x10^6$ cpm per ml in hybridization solution (500 µg/ml tRNA, 50 µM DTT, 50% formamide, 0.25 mM NaCl, 1X Denhardt's solution and 10% dextran sulfate). This hybridization solution was pipetted

on to the slides and incubated at 55°C overnight. Following hybridization, the slides were rinsed in 4X SSC, RNase treated (25 µg/ml RNase A for 30 min. at 37° C), rinsed in decreasing concentrations of SSC containing 1 mM DTT (final stringency at 0.1X and 70°C) and then dehydrated in ascending concentrations of ethanol. The slides were then exposed to ß-max film for 2-3 days before being dipped in Kodak NBT-2 emulsion. After 2 weeks of exposure at 4° C, the slides were developed in Kodak D-19 developer and counter stained with thionin. Alternating slides were collected and used to conduct the same survey with a sense riboprobe and with thionin staining alone.

3. Animals , Morphine Treatment, Analgesic and Behavioral Assays

The rats used in the *in situ* hybridization studies are described above. All the mice used in these studies were obtained from a breeding colony run by Dr. Malcolm Low. C57BL/6J male mice, all 7 weeks of age and 20-30 grams in weight, were made tolerant to morphine using a twice daily intraperitoneal (ip) injection paradigm (injection schedule and analgesic tests were essentially the same as those described by Ben-Bassar *et al.*, 1959). Briefly, the animals were tested for baseline tail withdrawal latency in a 49° C water bath and then injected ip with either 15mg/kg of morphine sulfate (Sigma) in 0.9% saline or saline alone. The animals were then measured for tail withdrawal latency 30 minutes after injection. This procedure was carried out between 9 AM and 11 AM on ten consecutive days. The animals received a second daily injection between 5 and 6 pm although tail withdrawal latency was not measured. After 10 days of this regimen the animals were sacrificed for dissection or subjected to behavioral tests.

On day 10 some of the animals were tested for physical dependence by challenging with an ip injection of naloxone. It had been determined that the mice were more susceptible to the effects of naloxone if the antagonist was administered 1-2 hours after their morning morphine injection. The degree of dependence achieved in the analgesically

tolerant mice was determined by counting the number of escape jumps, a classic opiate withdrawal symptom, following injection of 5 mg/kg naloxone.

In other experiments OFQ delivered icv was tested for its ability to precipitate withdrawal symptoms. Previous trials had suggested that the animal's response to OFQ was more robust if the peptide was administered before the morning injection of morphine. Mice were lightly anesthetized with isofluorane and then received a 2 µl icv injection of artificial cerebrospinal fluid (ACSF: 277 mM NaCl, 6.7 mM KCl, 4.5 mM $CaCl_2$, 3.0 mM $MgCl_2$, 2.9 mM NaH_2PO_4, 14.6 mM Na_2HPO_4, 5.4 mM Glucose, pH 7.4) with or without 0.15 nmoles of OFQ peptide. Thus, there were four experimental groups: morphine dependent mice receiving icv OFQ or ACSF alone and morphine naive mice receiving icv OFQ or ACSF alone. The injections were delivered by hand approximately 1 mm away from the midline of the skull and 2.5 mm behind the bregma suture using a Hamilton syringe attached to a 27 gauge needle fitted with a depth control guide (Laursen & Belknap, 1986).

After icv injection the animals were placed in a new cage with bedding and observed for withdrawal signs. The animals were also videotaped and later scored for total locomotion and rearing. Locomotion was measured by reviewing videotape on a television monitor fitted with a grid which divided the image of the cage into nine sections. A line cross was recorded whenever an animal moved all four paws from one grid section to another. After the behavioral testing animals were sacrificed and their brains were removed and fixed in 4% paraformaldehyde overnight. The success of the icv injection was verified by dissecting the brains under a dissecting microscope and tracing the path of the needle. Animals in success of injection could not be determined were excluded from the analysis. The differences in line crossing and rearing between the four experimental groups were compared statistically with a two-way ANOVA using Statistica software.

4. Dissections

The animals to be examined for OFQ peptide and ppOFQ mRNA levels were sacrificed by cervical dislocation one hour after the final morphine treatment and their brains were immediately removed. The dissections are described schematically in Figure 2 of Chapter 2. The brains were blocked into sections with three vertical cuts; the first cut passing through the optic chiasm, the second just caudal to the mammilary body and the third at the caudal end of the medulla.

In the most rostral section a pyramidal piece of tissue from the ventral forebrain (VBS) was removed. In this same section a small horizontal incision was made just above the corpus callosum which included sectors of the cingulate and motor divisions of the frontal cortex (FCX).

In the second block of tissue, two vertical incisions were made extending dorsally until even with the top of the third ventricle. A horizontal cut at the same level gave three pieces of tissue, one hypothalamic and the other two enriched for amygdala (AMG). The hypothalamus was then bisected into anterior and posterior portions (AHP and PHP respectively). Above the third ventricle the medial thalamus (MT) was peeled away from the hippocampus and cortex.

In the third block of tissue, the cerebellum was removed and two additional vertical slices were made at the rostral and caudal constrictions bordering the pons. Each of the resulting three pieces were then divided into dorsal and ventral sections. The most rostral pair of sections were enriched for the dorsal and ventral midbrain (DMB and VMB) respectively. The second set of sections contained dorsal (DP) and ventral (VP) nuclei at

the level of the pons. Finally, the most caudal section pair contained the dorsal and ventral medulla (DM and VM).

5. Radioimmunoassay

Radioimmunoassays (RIA) were performed as described by Quigley *et al.*, 1998. Briefly, tissues were weighed prior to homogenization in 10% acetic acid and 1% BSA. After lyophilization the samples were resuspended in 300 ml of assay buffer (50 mM $NaHPO_4$, 2.5 mg BSA 0.02% Na Azide and 0.2% ß-mercaptoethanol). A series of four two-fold dilutions for each tissue was incubated overnight with anti-OFQ antiserum at 4°C. The next day a competition assay for the antibody was set up by adding 10,000 cpm of iodinated OFQ peptide (generously supplied by Dr. Richard Allen). After removing the unbound peptide with charcoal, the amount of OFQ peptide present was determined by counting the antibody-bound fraction on a gamma counter and comparing to a standard curve generated by known concentrations of cold OFQ. The total peptide present was then normalized to the weight of the tissue. Morphine treated animals and saline control peptide levels were compared statistically with a one-way ANOVA using Statistica software.

6. Mouse cDNA Probes

A nearly full-length ppOFQ cDNA was generated using oligonucleotide primers based on the published sequence (Houtani *et al.*, 1996) and an RT-PCR strategy. A 244 base-pair Pst I fragment encoding the deduced amino acid sequence for the mature OFQ peptide was subcloned into pBluescript from which the sense (T7 polymerase) and antisense (T3 polymerase) riboprobes were generated. A 309 base-pair PCR fragment was generated with primers designed from the partial mouse OFQR sequence published by Mollereau *et al.*, 1996 and subcloned into pAMP10 such that antisense (T7 polymerase) and sense (SP6 polymerase) riboprobes could be synthesized. Finally, a 158 bp PCR fragment of cyclophilin (CYC) cloned into pGEM4 was used as an internal loading control.

7. RNase Protection

Radioactive antisense riboprobes were synthesized using 100 µCi of ^{32}P-UTP and the appropriate polymerase essentially as described in Current Protocols of Molecular Biology (Ausubel *et al.*, 1987). Full length riboprobes were purified using a Full Lengther apparatus (Cascade Biologicals). Twenty µg of total RNA, isolated from the tissues described above using TRIZOL reagent (GIBCO-BRL), was resuspended in a 30 µl hybridization cocktail containing 4 x 10^5 cpm of OFQ, 2 x 10^5 cpm of OFQR and 2000 cpm CYC and incubated overnight at 42 ^{o}C. After treatment with RNase the protected fragments were run on a 6% acrylamide gel with 8M urea and 0.5X TBE. The gels were dried and bands were visualized by exposing the gel on a Phosphoimager (Molecular Dynamics). The appropriate bands were quantitated densitometrically using IP-LabGel software (NIH). Relative RNA levels were determined by normalizing OFQ and OFQR to CYC. Morphine treated animals and saline control mRNA levels were compared statistically with a one-way ANOVA using Statistica software.

8. Quantitative Analysis of *In Situ* Hybridization

In some experiments *in situ* hybridization was used to compare OFQ mRNA expression between three morphine dependent and three naive mice. In these experiments *in situ* hybridization was performed as described above. Every section of each brain was mounted consecutively on threes sets of slides. The first set was to be used for OFQ mRNA analysis, the second for OFQR mRNA analysis and the third to repeat any sections as necessary. After the hybridization procedure the slides were then exposed to ß-max film (Amersham) for 10 days before being dipped in Kodak NBT-2 emulsion. After 2 weeks of exposure at 4^{o}C, the slides were developed in Kodak D-19 developer and counter stained with thionin. Quantitation was performed by analyzing the autoradiographs densitometrically using IP-LabGel software. Morphine-treated animals and saline control

mRNA levels were compared statistically with a one-way ANOVA using Statistica software.

Chapter 1

Localization of ppOFQ and OFQR mRNA: an in situ hybridization survey

This work is published in two articles:

The Orphanin FQ System: A New Emerging Target for the Management of Pain

Tristan Darland and David K. Grandy (1998). British Journal of Anaesthesia (in press)

Orphanin FQ/Nociceptin: a role in pain and analgesia, but so much more

Tristan Darland, Mary M. Heinricher and David K. Grandy (1998). Trends in Neuroscience (in press)

1. Introduction

The heptadecapeptide orphanin FQ (OFQ), also called nociceptin, is the endogenous ligand for the orphan receptor LC132 (also referred to as the opioid receptor-like protein, ORL-1) was purified from whole rat brain (Meunier *et al.*, 1995) and porcine hypothalamic extracts (Reinscheid *et al.*, 1995). Efforts to clone the gene encoding OFQ revealed that it is synthesized as a larger precursor protein (ppOFQ) that is cleaved into multiple peptides in a manner reminiscent of the endogenous opioid ligands (Mollereau *et al.*, 1996; Nothacker *et al.*, 1996). The amino acid sequence of the OFQ peptide is most closely related to the opioid dynorphin. Despite its structural similarities, OFQ does not bind or activate the classic opioid receptors (Reinscheid *et al.*, 1995). Similar to the opioid receptors, the OFQ receptor (OFQR) acts in a Gi/Go-coupled fashion upon binding of ligand, inhibiting adenylyl cyclase, activating inwardly rectifying potassium channels (Conner *et al.*, 1996; Ikeda *et al.*, 1997; Vaughn & Christie, 1996; Vaughn *et al.*, 1997; Wagner *et al.*, 1998) and inhibiting voltage-sensitive calcium channels (Connor *et al.*, 1996; Knoflach *et al.*, 1996). OFQR does not bind any of the classic opioid receptor agonists or antagonists with significant affinity (Reinscheid *et al.*, 1995).

While OFQR's structure, sequence and G-protein coupling are similar to the classic opioid receptors, the actions of OFQ administration mediated by OFQR at the behavioral level are quite different. Based on earlier studies it was suggested that intracerebroventricularly (icv) administration of OFQ induced hyperalgesia (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995). However, these studies were confounded by the absence of an uninjected control group. Subsequent studies have suggested that in mice an icv injection under anesthesia may cause an opioid-mediated stress-induced analgesia that is opposed by OFQ (Mogil *et al.*, 1996b). What is certain is that OFQ administered icv functionally antagonizes several of morphine's effects in mice and rats including analgesia,

hypothermia and Straub tail (Mogil *et al.*, 1996b; Tian *et al.*, 1997a). In addition, OFQ has recently been shown to stimulate feeding in satiated rats (Pomonis *et al.*, 1997; Stratford *et al.*, 1997); has potent anxiolytic properties (Jenck *et al.*, 1997); and the OFQR has been implicated in the regulation of the auditory system (Nishi *et al.*, 1997). Further study is likely to reveal additional behavioral roles for OFQ especially now that an antagonist is available (Guerrini *et al.*, 1998).

In an effort to form rational hypotheses about the potential roles of the OFQ-OFQR system, we have performed an extensive survey of ppOFQ and OFQR mRNA synthesis in the rat CNS by *in situ* hybridization. Using *in situ* and immunohistochemical evidence of OFQR expression (Anton *et al.*, 1996) we have attempted to map some of the circuitry in the rat CNS that may involve OFQ and then speculate about some of the peptide's possible functions.

2. Results

Expression of ppOFQ and its Receptor in the Rat Central Nervous System

In addition to revealing that OFQ is processed from a precursor molecule the cloning of the rat ppOFQ cDNA has provided valuable *in situ* hybridization probes. These probes have made possible the detailed mapping of ppOFQ and OFQR expression throughout the central nervous system that has led to a better understanding of the peptide's functions in the central nervous system (Darland & Grandy, 1998; Darland *et al.*, 1998; Houtani *et al.*, 1996; Nothacker *et al.*, 1996).

The telencephalon

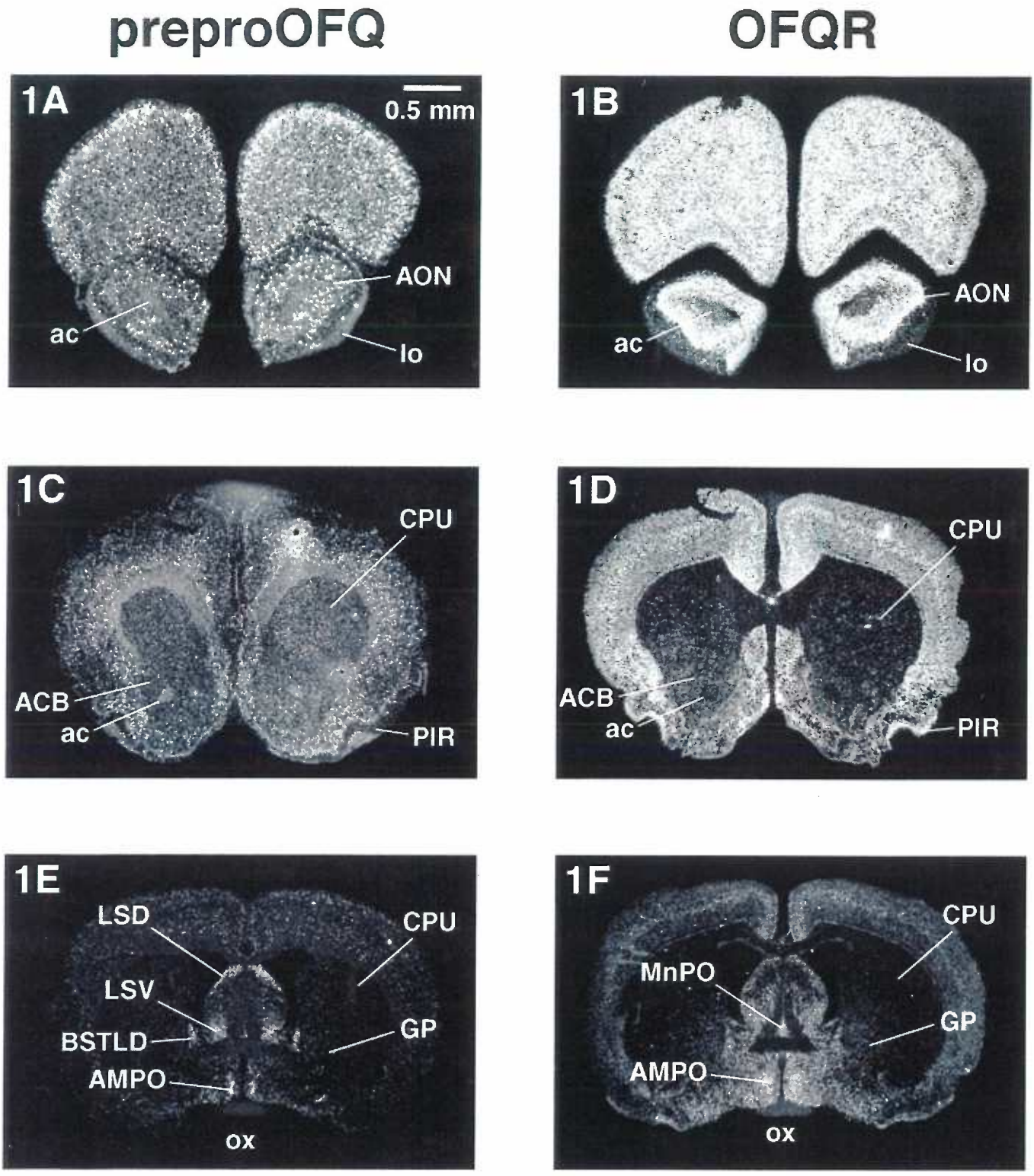
There is expression ppOFQ mRNA in the cerebral cortex, wherethe density of hybridization is greatest in the superficial, rather than deeper layers (Figures 1-3). In contrast, OFQR mRNA expression is moderate in cortex and is particularly dense in the

intermediate layers. The expression pattern for both the precursor and the receptor appears to be uniform along the rostral-caudal axis of the cortex. However, in the piriform cortex there is no ppOFQ mRNA expression while OFQR mRNA expression is very dense (Figure 1C and ID). The cortical regions of the olfactory bulbs display moderate expression of both precursor and receptor (Figure 1A and 1B). In the anterior olfactory nucleus moderate expression of ppOFQ and very dense expression of the receptor were detected in all divisions (Figure 1A and 1B).

In the medial and central divisions of the amygdala (AMG), the lateral ventral and lateral dorsal septal nuclei, the anterior and medial posterior divisions of the bed nucleus of the stria terminalis (BST) and the medial preoptic area (POA) the expression of ppOFQ mRNA is dense (Figure 1E and Figure 2A, 2C and 2E). The receptor mRNA shares a similar distribution pattern to the precursor in the AMG, BST and POA (Figure 1F and Figure 2B, 2D and 2F). However, unlike the precursor, expression of OFQR mRNA in the lateral septum is only moderate while expression in the medial divisions is dense.

In the hippocampus ppOFQ probes produced moderate labeling of CA1, CA2 and CA3 hippocampal neurons but the signal was considerably less in neurons of the dentate gyrus (Figure 2C and 2E). In contrast to the moderate expression of the precursor expression of OFQR mRNA is extremely dense throughout the hippocampus (Figure 2D and 2F).

In the striatum a ppOFQ probe lightly labeled the ventral pallidum and globus pallidus while the caudate putamen and nucleus accumbens were conspicuously devoid of ppOFQ mRNA (Figure 1C, 1E and 2A). These areas displayed a similar expression pattern for receptor mRNA (Figure 1D, 1F and 2B).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LSD
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LSV
BSTLD
GP
AMPO
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1F
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MnPO
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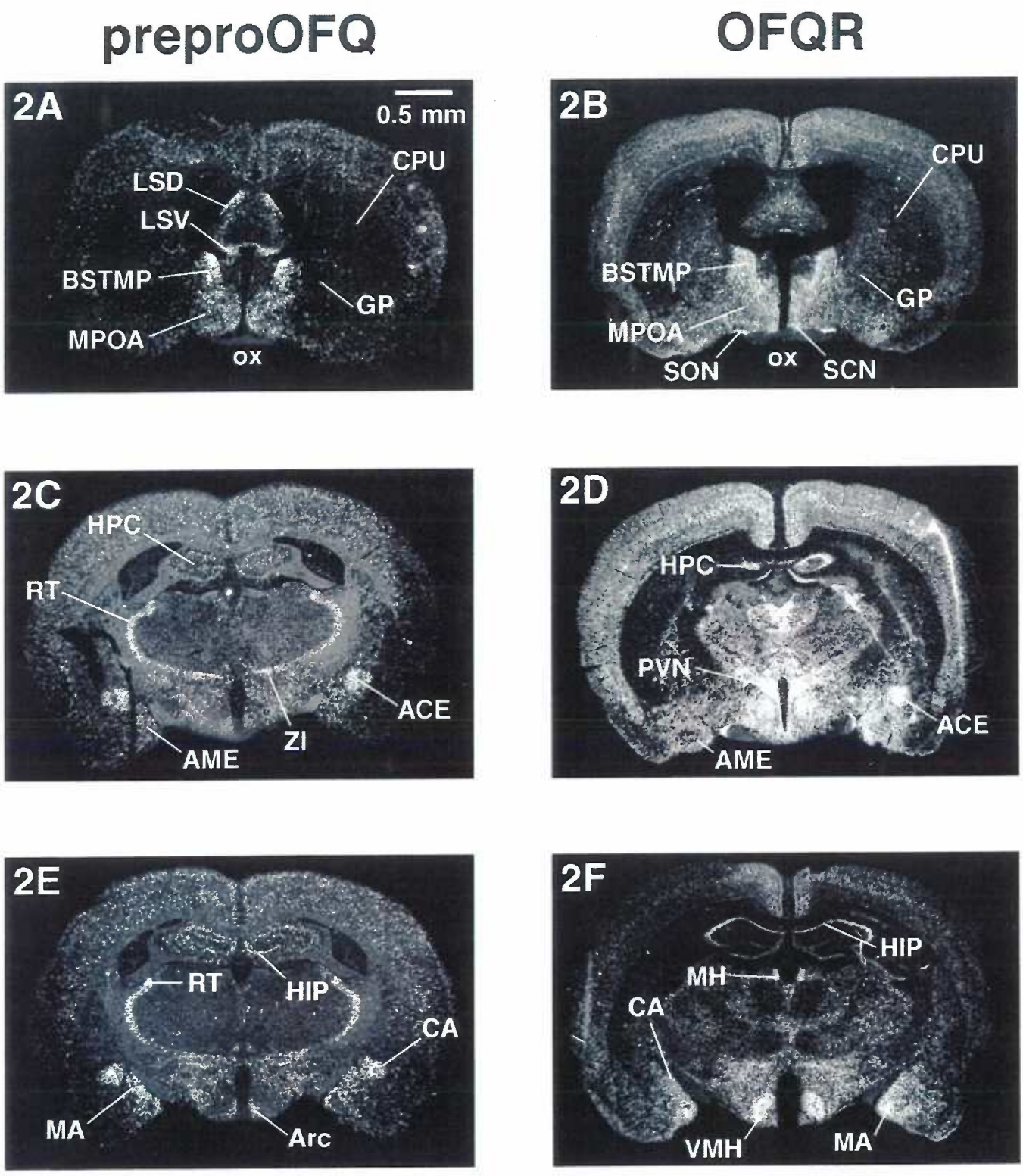
AMPO
ox

Figure 1

The diencephalon

Several thalamic nuclei displayed ppOFQ mRNA expression including the subparafascicular and reticular thalamic nucleus, both of which were particularly dense (RT, Figure 2C and 2E). The paratenial and central medial nuclei of the thalamus were only lightly labeled in the anterior regions. The ventral lateral geniculate and intergeniculate leaflet displayed moderate staining (Figure 3A) while the dorsal lateral and medial geniculate appeared to be negative for ppOFQ as were the habenula, the ventrolateral, ventromedial and paraventricular thalamic nuclei. Expression of OFQR mRNA was dense in the medial habenula and moderate in the lateral habenula, reuniens thalamic nucleus and zona incertia (Figure 2F). In contrast to the pattern displayed to the precursor, many nuclei of the medial thalamus displayed light expression for OFQR mRNA including the paraventricular, anterodorsal, centromedial, ventromedial, laterodorsal and ventroposterolateral thalamic nuclei (Figure 2D and 2F). The ventrolateral, medial dorsal, centrolateral, gelatinosus and posterior ventral thalamic nuclei were conspicuously void of OFQR mRNA expression.

Although moderate expression of ppOFQ mRNA was detected in the paraventricular nucleus (PVN), the dorsomedial nucleus and the lateral area of the hypothalamus the arcuate nucleus (ARC) and the zona incerta (ZI) and anterior hypothalamic area were densely labeled (Figure 2A, 2C and 2E). Conspicuously lacking ppOFQ mRNA were the ventral medial nuclei of the hypothalamus (VMH), the suprachiasmatic (SCN) and supraoptic nuclei (SON) (panels 2A and 2C). The mammillary nuclei and the pituitary were also negative with respect to detectable ppOFQ expression (data not shown). In contrast, the VMH and ARC displayed very dense expression of OFQR mRNA while the SCN, SON and the PVN displayed dense expression of the receptor mRNA (Figure 2B, 2D and 2F). There was moderate expression of OFQ mRNA in the dorsomedial and lateral hypothalamic area and light expression in the mammillary nuclei.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AME
2D
HPC
PVN
ACE
AME
2E
RT
HIP
CA
MA
Arc
2F
HIP
MH
CA
VMH
MA

Figure 2

The mesencephalon

In the midbrain ppOFQ mRNA is moderately expressed in the periaqueductal gray (PAG) and the superficial layer of the superior colliculus whereas all layers of the inferior colliculus were lightly labeled (Figure 3C and 3E), as were the ventral tegmental area (VTA) and substantia nigra pars compacta (SNC) (Figure 3A). The interpeduncular nuclei displayed moderate ppOFQ mRNA but no ppOFQ expression was detected in the substantia nigra pars reticulata (SNR) or the red nucleus. A similar pattern for OFQR mRNA expression was detected in the PAG and superior colliculus (Figure 3 panels D and F). In contrast, the VTA was densely labeled for receptor message while the SNc and interpeduncular nuclei were moderately labeled.

The pons, medulla and cerebellum

The medioventral, lateroventral and superior periolivary nuclei in the brainstem all displayed very dense expression of the ppOFQ mRNA as did the posterodorsal tegmental nucleus and the prepositus hypoglossi (PrH) (Figure 4A and 4C). The raphe magnus (RM) locus coereleus (LC), solitary tract and spinal trigeminal nuclei all demonstrated moderate labeling (Figure 4A and 4E). Interestingly, no ppOFQ mRNA was detected in the nucleus paragigantocellularis, dorsal raphe (RD), pontine, or cochlear nuclei (Figure 3E and 4C). In the cerebellum only the medial cerebellar nucleus appeared to express ppOFQ mRNA (Figure 4C). Receptor mRNA was also widely distributed in the brainstem with very dense expression detected in the LC and RD (Figure 3F and 4B). The pontine, vesibular, cochlear, solitary tract, lateral reticular, median raphe, RM, sensory and spinal trigeminal nuclei displayed moderate expression (Figure 3F, 4B, 4D and 4F). Moderate OFQR mRNA expression was also detected in the medial and interposed cerebellar nuclei (Figure 4D). Only light expression, if any, was detected in the periolivary regions.

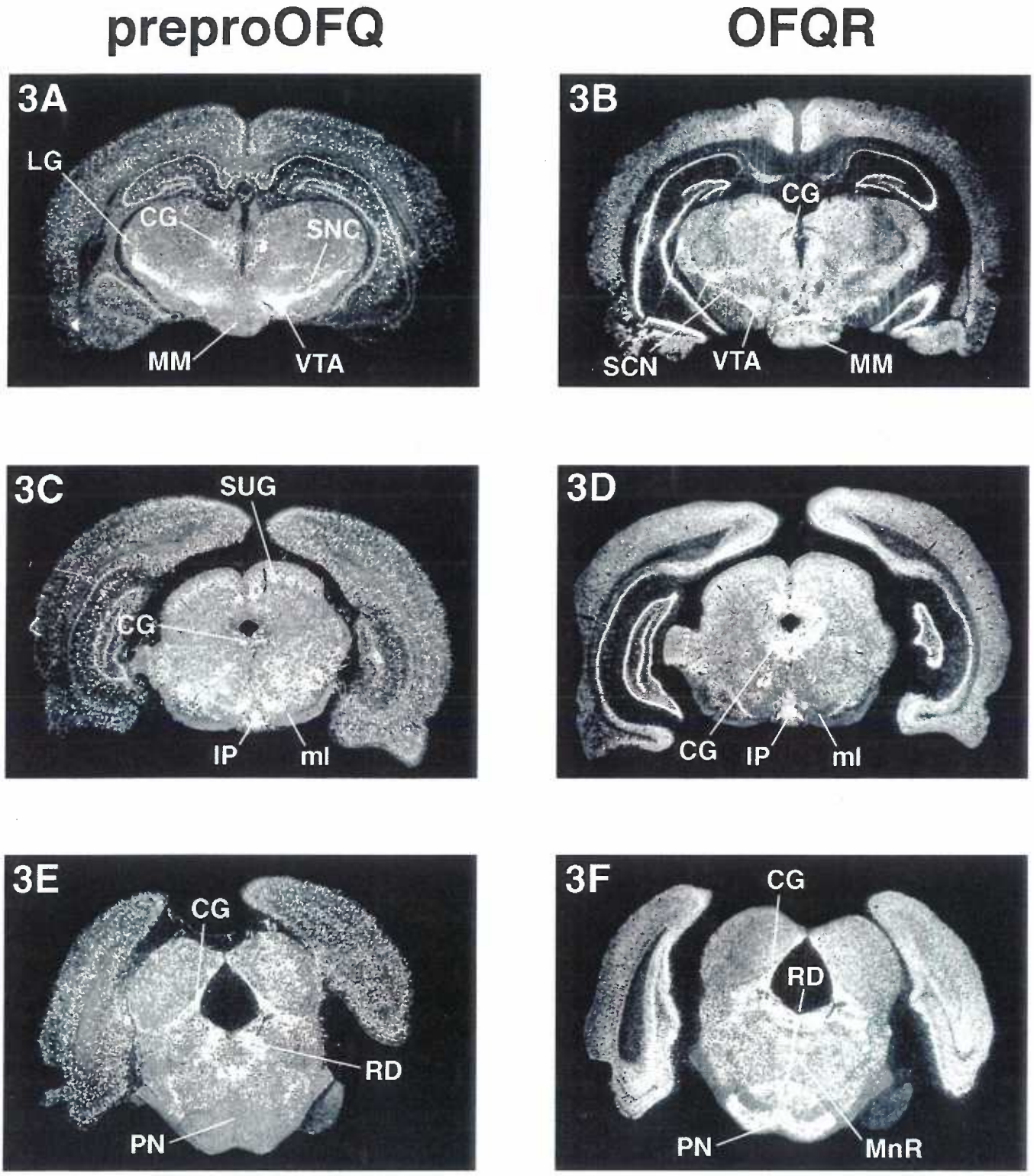
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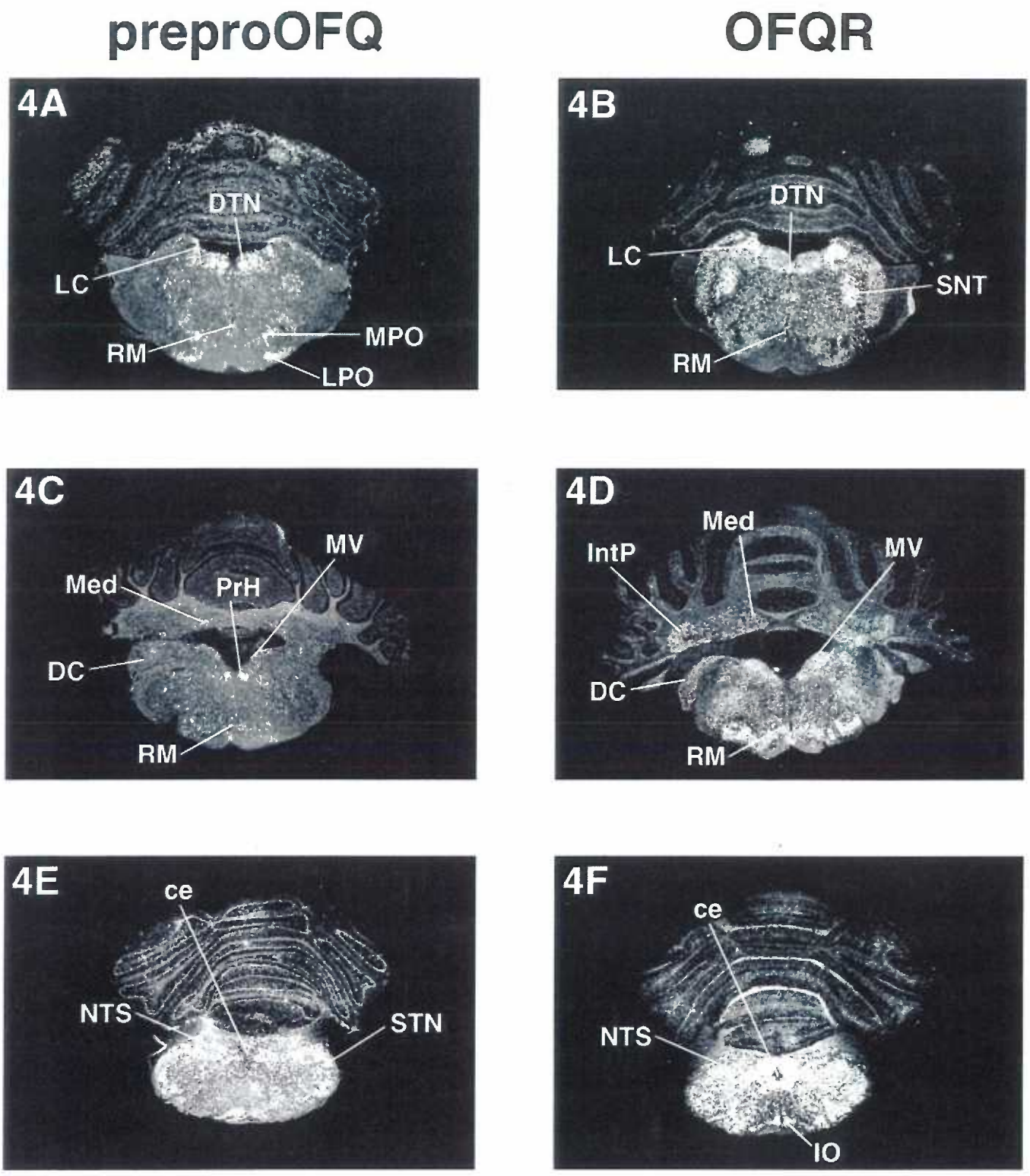
Figure 3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4C
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PrH
DC
RM
4D
Med
IntP
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4E
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NTS
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Figure 4

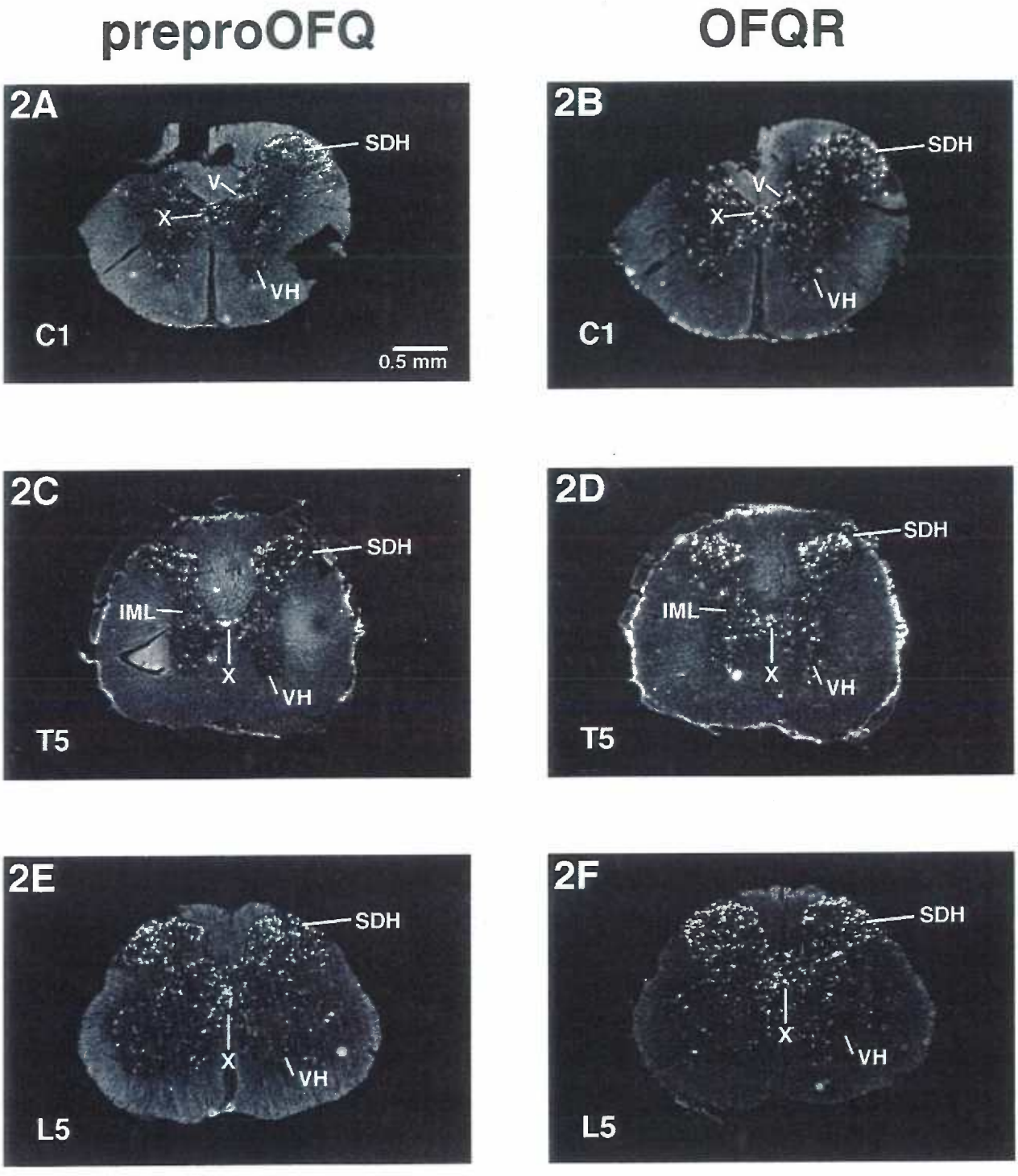
The spinal cord

The expression pattern of ppOFQ mRNA has also been examined in the cervical, thoracic and lumbar segments of the rat spinal cord. While some diffuse signal was detected centrally in all segments examined, the most intense hybridization to ppOFQ mRNA was confined to the superficial layers of the dorsal horn corresponding to lamina (l.) I and II (Figure 5). It is noteworthy that in cervical sections of the cord very little hybridization corresponding to either ppOFQ or the OFQ receptor mRNA was detected in l.V, although some diffuse signal can be seen in l.X. In more caudal regions of the cord hybridization to ppOFQ transcripts is seen in the superficial layers of the dorsal horn, l.V and l.X, as well as scattered cells in l.VII of the ventral horn (Figure 5E).

Spinally the receptor's mRNA is expressed throughout the length of the cord (Figure 5B, 5D and 5E). In the cervical segments orphan receptor mRNA is most abundant in the superficial layers of the dorsal horn corresponding to l.I and l.II as well as l.X. The distribution of the receptor's mRNA in the cervical cord is maintained as one moves more caudal into the thoracic cord. Changes in the distribution of OFQR mRNA begin to appear in segments of the lumbar cord where, in addition to labeling the superficial layers of the dorsal horn, a significant amount of receptor mRNA is scattered throughout l.VII of the ventral horn (Figure 5F).

3. Discussion

The expression patterns of ppOFQ and its receptor are summarized in the schematic shown in Figure 6. Knowledge of these expression patterns has spearheaded experimental efforts to determine the functions of the OFQ peptide. The following discussion reviews some of these efforts.


preproOFQ
OFQR
2A
SDH
V
X
VH
C1
0.5 mm
2B
SDH
V
X
VH
C1
2C
SDH
IML
X
VH
T5
2D
SDH
IML
X
VH
T5
2E
SDH
X
VH
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2F
SDH
X
VH
L5

Figure 5

Distribution of prepro Orphanin FQ (ppOFQ) and OFQ Receptor (OFQR) mRNAs

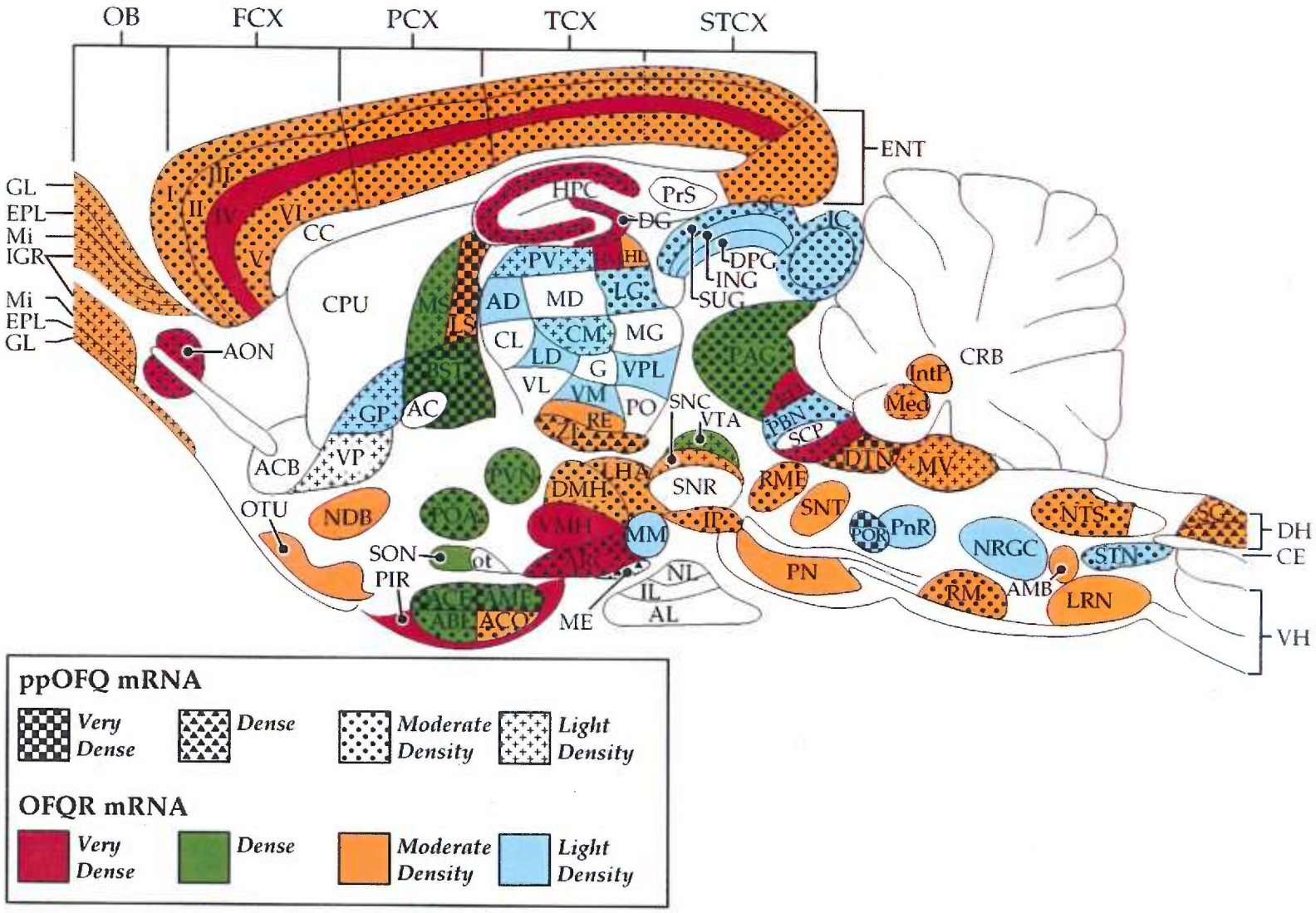

Figure 6

Limbic regulation

Moderate expression of mRNA for both ppOFQ and its receptor suggest a role in cortical function. OFQ decreases glutamatergic release in perfused cerebrocortical slices and therefore is likely to modulate cortical processing (Nicol *et al.*, 1996). This function is not limited to any particular sensory modality, as the expression pattern appears uniform along the rostral-caudal axis.

The POA, BST and AMG are sexually dimorphic areas and as such are subject to regulation by sex hormones (reviewed by Segovia & Guillamon, 1993). The finding that both the ppOFQ and its receptor mRNAs are intensely expressed in these areas suggests a possible role for the OFQ system in the expression of sexually dimorphic behavioral responses, including perhaps responsiveness to opiates.

Dense expression of OFQR mRNA, as well as moderate expression of the precursor, in the hippocampus suggests a role for the OFQ system in learning and memory . Indeed, it has been reported that rats were found to have impaired spatial learning in the Morris water task, without decreased swimming performance, after injection of OFQ into the CA3 region of the dorsal HIP (Sandin *et al.*, 1997).

Thalamic Regulation

Dense expression of ppOFQ in the reticular thalamus (RT) is interesting because it has reciprocal connections with most, if not all, of the other thalamic nuclei. It is also thought to be responsible for the rhythmic modulation of corticothalamic neuronal electrical activity which has been found to be correlated with the animal's level of vigilance (Steriade *et al.*, 1986). The output of the RT is inhibitory and most of its neurons, at least in rat, are

GABAergic. Therefore, future studies might explore the effect of OFQ on the activity on thalamic neurons during different states of vigilance.

Hypothalamic regulation

Dense expression of OFQR in certain nuclei of the hypothalamus points to several potential functions for the peptide. Since the VMH is thought to be an area involved in reproductive behavior it is possible that OFQ, originating in other sexually important areas such as the BST, may participate in regulating output from the VMH. In fact, it has been demonstrated recently that OFQ injected into the VMH dose dependently facilitates lordosis in female rats (Sinchak *et al.*, 1997). The VMH is also recognized as an important area that may regulate feeding behavior, a hypothesis that recently received some support when it was demonstrated that OFQ injected into the VMH stimulated feeding in sated rats (Pomonis *et al.*, 1997; Stratford *et al.*, 1997).

With respect to the PVN of the hypothalamus this nucleus is an important source of corticotropin-releasing hormone (CRH) which is involved in the regulation of the hypothalamic-pituitary-adrenocortical (HPA) stress axis (Grino *et al.*, 1989). Afferents to the PVN include those originating in the lateral septum (LS), the BST and the POA (reviewed by Herman & Cullinan, 1997). These areas are thought to be involved in the production of fear and anxiety (Gray *et al.*, 1993) and given that ppOFQ mRNA is heavily expressed in these projecting areas this peptide may well be a new modulator of the HPA axis. In fact, it has recently been demonstrated the OFQ has potent anxiolytic properties in several behavioral assays (Jenck *et al.*, 1997).

Dense expression of the OFQR has been reported in the SCN where its activation is coupled to inwardly rectifying K^+ channels (C. Allen et al., in preparation), suggesting that another possible role for OFQ may be the regulation of circadian rhythms. Indeed, Allen et

al. found that OFQ injected into the SCN of hamsters alters the light-induced phase-shift in circadian wheel running behavior. This hypothetical role for OFQ in modulating circadian rhythms is further supported by our observation that ppOFQ mRNA is expressed in the LG and IGL, areas that send fibers to the SCN which are thought to regulate the light-sensitive aspects of circadian rhythm. Neuropeptide Y (NPY) has also been implicated as an important neurotransmitter in this regulation (Shinohara *et al.*, 1993), however, the leaflet sends many fibers to the SCN which are NPY negative. Interestingly ppOFQ mRNA is not detectable by *in situ* hybridization in the SCN. Therefore, if OFQ is involved in circadian regulation, its source in vivo could be the IGL where it may act to modify the NPY input to the SCN. Alternatively, the retina may provide another source of the peptide for the SCN. Interestingly, ppOFQ mRNA has been detected in the retina by RNase protection (our unpublished results). Furthermore, OFQ peptide inhibits light-induced acetylcholine release from amacrine cells (Neal *et al.*, 1997).

The abundant expression of ppOFQ mRNA in the ARC nucleus raises several interesting possibilities given the complex nature of its connections. Recently it was demonstrated that OFQ receptors are functional in GnRH positive neurons of the ARC (Wagner *et al.*, 1998) suggesting the possibility that OFQ may modulate the pulsatile release of this important reproductive hormone. Wagner et al. have also demonstrated the presence of functional OFQ receptors on tyrosine hydroxylase (TH) positive and ß-endorphin positive neurons in the ARC. Therefore, it is likely that OFQ affects dopamine neurons that influence the pituitary and ultimately prolactin secretion as well as opioid neurons that project throughout the brain.

Localization of ppOFQ in the ARC, as well as in many other areas of the hypothalamus, also suggests the possibility that in addition to being a *bone fide* neurotransmitter the peptide might also act as a releasing factor. At this time we consider

this possibility unlikely because we have found no expression of the receptor in the pituitary. However, it is possible that OFQ or one of the other peptides processed from the precursor may either regulate the secretion of releasing factors by the hypothalamus or be released into the blood stream where it could act as a hormone or modulator of the immune system. In fact, there is evidence that OFQ may act in the circulation. For example, it has been reported that OFQR is functionally expressed in lymphocytes (Halford *et al.*, 1995). It has also been shown recently that OFQ possesses vasorelaxant properties (Champion & Kadowitz, 1997) and affects renal excretion of water and sodium in rats (Kapusta *et al.*, 1997).

Dense expression of OFQR in the SON and subfornical organ (SFO) suggests a possible role for OFQ in regulating angiotensin, vasopressin and oxytocin release and therefore, water balance and blood pressure. Kapusta et al. demonstrated that OFQ administered icv produced selective water diuresis . Potential OFQ afferents to the SON and SFO include the nuclei of the solitary tract which may relay information from the periphery, the raphe nuclei and the POA (Sawchenko & Swanson, 1983). With combined effects, centrally and peripherally, OFQ may therefore prove to be very important in regulating water balance and ultimately blood pressure.

Monoamine regulation

The LC, the major noradrenergic nucleus in brain, displays high levels of OFQ receptor mRNA (Figure 3) and protein (Anton *et al.*, 1996). A high level of expression for the precursor mRNA has also been detected in the PrH (Figure 4), one of the two major afferents to the LC. The other major LC afferent, the nucleus paragigantocellularis, was negative for ppOFQ mRNA. Therefore, based solely on the *in situ* hybridization data it seems likely that OFQ will be found to be a major modulator of LC output in vivo. In fact OFQ has been shown to potently hyperpolarize neurons in the LC (Conner *et al.*, 1996).

The fact that the LC is important for determining the arousal state of the animal it suggests that OFQ acting on this nucleus may have sedative effects.

Several lines of evidence suggest that OFQ affects the dopaminergic (DA) neurons that originate in the ventral midbrain and have been implicated in stimulated reward behavior. First, OFQR mRNA is expressed at relatively high levels in neurons of the VTA (See figure 3). Second, OFQ hyperpolarizes tyrosine hydroxylase (TH) positive cells in brain slices (A. Bonci unpublished communication). Third, when injected into rats cannulated in the VTA, OFQ blocks morphine-stimulated locomotor activity in a dose-dependent manner (P. Kalivas unpublished communication). Finally, when injected icv, OFQ has been reported to decrease dopamine release in the nucleus accumbens.(Murphy *et al.*, 1996).

Where does this OFQ in the VTA come from? The VTA receives input from several OFQ positive brain regions including the BST, AMG, CX and the VP. Studies using retrograde labeling in combination with *in situ* hybridization will be needed to clarify this point. Our data also suggest that at least a portion of the OFQ acting on receptors expressed by VTA originate there. However, the possibility that OFQ acts presynaptically on VTA projection neurons to regulate DA release needs to be examined further.

Other nuclei including the RD and the RM contain monoaminergic neurons which express both OFQ and OFQR. The AMG and POA have reciprocal connections with the BST as do the VTA, LC, DR and RM (Swanson & Cowan, 1979). Although much remains to be done, based on these findings we propose that one role of OFQ, and/or the other peptides processed from ppOFQ, may be to regulate and coordinate the firing of the major monoaminergic nuclei thereby linking vigilance with the emotional and motivational states of the animal.

Auditory regulation

The dense labeling of the periolivary regions (POR) by ppOFQ probes suggests that the peptide may be important in regulating the auditory system. In this regard it is interesting to note that OFQ receptor-deficient mice have a substantially decreased ability to adapt their auditory brainstem response upon exposure to intense sound (Nishi *et al.*, 1997). The ppOFQ mRNA-positive nuclei of the POR project to important relay nuclei in the auditory system which express OFQ receptor mRNA. The most important of these are the inferior olive, medial vestibular nucleus and the dorsal and lateral cochlear nuclei (data not shown). It remains to be determined whether OFQ exerts its effects on the auditory system via these nuclei or perhaps by acting directly on the hair cells of the cochlea since ppOFQ mRNA is expressed in areas where some of the neurons project directly to the outer hair cells (Brown, 1993).

Regulation of nociception

The dense expression of ppOFQ mRNA, as well as it receptor mRNA, in areas of the dorsal horn of the spinal cord that are known to be involved in the transmission of noxious information strongly suggests a role for the OFQ system in modulating nociceptive processing. It has been reported that activity of the superficial dorsal horn is inhibited by OFQ (Faber *et al.*, 1996; Liebel *et al.*, 1997; Stanfa *et al.*, 1996; Wang *et al.*, 1996). In addition, it has been demonstrated that OFQ is capable of producing analgesia when administered intrathecally (Garaulet *et al.*, 1995; Grisel *et al.*, 1996; King *et al.*, 1997; Tang *et al.*, 1984a; Tian *et al.*, 1997a). The majority of primary nociceptive afferents terminate in l.I, l.II and l.V (Cervero & Iggo, 1980). Many neurons in these regions are nociceptive and project rostrally to the reticular formation, thalamus and other parts of the forebrain. It is not yet known whether the spinal neurons expressing OFQ project rostrally. However, the anatomical observation that very little OFQR mRNA or protein is seen in the ventral basal and posterior thalamus argues against expression in spinothalamic

neurons (our results and Anton *et al.*, 1996). Therefore the neurons expressing OFQ in the dorsal horn are likely to be spinoreticular projection neurons or interneurons within the spinal cord. It is also worth noting that essentially no labeling of receptor mRNA is seen in the intermediolateral nucleus (IML) which contains preganglionic sympathetic neurons that are primarily involved in autonomic control (reviewed by Gebber & McCall, 1976).

OFQ is known to play an important role in pain modulation and nociception at supraspinal sites as well. In behavioral assays of nociceptive sensitivity OFQ administered supraspinally has been shown to antagonize the analgesic effects of stress (Mogil *et al.*, 1996b), morphine (Mogil *et al.*, 1996b; Tian *et al.*, 1997a) and electroacupuncture (Tian *et al.*, 1997b). Although the exact brain structures responsible for these responses remain to be identified the PAG is currently an attractive candidate. In addition to its role in various behaviors, the PAG is widely recognized as an important area of the brain in terms of nociceptive processing. This is based, in part, on the ability of electrical stimulation of the PAG to produce analgesia in animals and relieve severe pain in humans and on the realization that opioids are capable of producing profound analgesia when administered into the PAG (Hosobuchi *et al.*, 1997). Evidence for OFQ regulation of PAG function include reports that OFQ inhibits the firing of neurons in the PAG via both pre- and post-synaptic actions (Vaughn *et al.*, 1997) together with the observation that the peptide can have profound behavioral consequences with respect to an animal's response to morphine (Morgan *et al.*, 1997).

Another brain region that has now been shown to be exquisitely sensitive to the effects of exogenous OFQ is the rostral ventral medulla (RVM) (Heinricher *et al.*, 1997). The demonstration that OFQ potently blocks the analgesic effect of opioids when locally administered to the RVM is consistent with the peptide's ability to suppress the firing of "off-cells", a population of neurons that when disinhibited by opiates interfere with

nociceptive processing in the dorsal horn of the spinal cord (Fields *et al.*, 1991; Heinricher *et al.*, 1994). Clearly OFQ's role in modulating nociception involves the coordinated activity of multiple areas of the central nervous system.

Potential role in development of morphine tolerance and dependence

The expression pattern of OFQ and its receptor as well as the peptide's effects on the actions of morphine suggest a role in the development of tolerance and dependence. The LC, PAG, RM, hypothalamus and amygdala have all been implicated as neural substrates of opiate dependence (Maldonado *et al.*, 1992). All these areas show strong expression of OFQ, its receptor or both together. It has long been suggested morphine tolerance and dependence develop from a combination of desensitization of opioid receptors and upregulation of other neuropeptide systems functionally antagonistic to the opioid system (reviewed by Smith *et al.*, 1989). OFQ may be one of these antagonistic systems.

Our studies have shown that OFQ peptide levels increase in the ventral brainstem and hypothalamus of morphine-dependent mice (see chapter 2). Interestingly, this increase in peptide correlates with an increase in OFQ mRNA in the basal forebrain complex including the POA, LS and BST. This complex, particularly the BST, sends extensive efferents to the hypothalamus, most notably the PVN, and moderately dense efferents to the VTA and regions of the brainstem including the LC, RD, RM and PAG (Swanson & Cowan, 1979). If OFQ is expressed in these efferent fibers then the peptide could potentially coordinate the reward, hormonal and autonomic aspects of morphine dependence. The basal forebrain has also received some attention of late as being involved in some of the affective aspects of drug dependence (Koob *et al.*, 1989; Stinus *et al.*, 1990). It is interesting that the BST-POA complex has been implicated as a neural substrate in disorders associated with anxiety and fear including post traumatic stress syndrome (PTSD) and depression (reviewed by

Herman & Cullinan, 1997)). Given its widespread distribution the OFQ system is likely to be an important molecule clinically.

Chapter 2

A potential role for OFQ in the development of morphine tolerance and dependence

This work is in preparation for submission

1. Introduction

Morphine has been the drug traditionally chosen by physicians to alleviate severe and chronic pain. Therefore, the potential side effects of chronic morphine use, such as the development of tolerance and dependence, have become major clinical concerns. The term tolerance is usually defined as the decreased effectiveness of the drug after repeated exposure. Physical dependence is a state that can be reached after chronic opiate exposure such that repeated use is required to prevent the onset of certain physical withdrawal signs, which in humans includes hot-cold flashes, increased perspiration, heart rate and respiration. In addition, a number of affective or motivational withdrawal signs such as anxiety, restlessness and irritability usually develop (Jaffe, 1990). These motivational signs of opiate abstinence are considered particularly important in the return to compulsive use as well as drug craving (Jasinski *et al.*, 1985).

Mice readily self-administer morphine, develop both tolerance and dependence and therefore serve as an excellent model system in which to study various aspects of opiate action (reviewed by Bhargava, 1994; Emmett-Oglesby *et al.*, 1990). While mice become less responsive to the locomotor, euphoric and respiratory effects of morphine after chronic exposure it is their decreased analgesic response that is most frequently used as a measure of tolerance. Opiate dependence in mice is most often determined by challenging the animal with an opioid receptor antagonist such as naloxone and measuring withdrawal symptoms such as jumping, writhing, wet-dog shakes, teeth chattering and diarrhea (Way *et al.*, 1973). In addition, several behavioral indices have been used to measure the affective or motivational aspects of opiate withdrawal in rodents including aversion to locations where abstinence occurred (Hand *et al.*, 1988; Manning & M.C. Jackson, 1977; Mucha, 1987), disruption of trained operant behavior (Gellert & Sparber, 1977), and suppression of exploration as measured by either activity in an elevated plus maze or an open field (Brady *et al.*, 1989; Higgins & Sellers, 1994; Schulteis *et al.*, 1994).

Morphine exerts its effects by binding endogenous opioid receptors in the central nervous system and periphery. These receptors couple negatively to adenylyl cyclase via G_i/G_o proteins (Childers, 1991), to inwardly rectifying potassium currents and to voltage-gated calcium channels (Gross *et al.*, 1990; North & Williams, 1983). Several studies in cell lines and isolated tissues have shown that the phenomenon of morphine tolerance appears to occur at the level of the opiate receptor in a two step process involving first a rapid desensitization, or uncoupling of the receptor from the signal transduction machinery, and second a long term reduction in the number of available receptors (reviewed by Johnson & Fleming, 1989; Smith *et al.*, 1989). Morphine dependence has also been characterized in these systems as an adaptation of cells to chronic morphine exposure such that abstinence or blockage with an antagonist results in the increased production of cAMP.

In spite of their usefulness cell culture and isolated tissue models fail to account for several aspects of morphine tolerance and dependence that are seen *in vivo*. For example, although receptor protein and mRNA downregulation has been demonstrated in tolerant animals (Bhargava & Gulati, 1990; Diaz *et al.*, 1995; Nishino *et al.*, 1990; Ronnekleiv *et al.*, 1996; Tao *et al.*, 1987; Tempel *et al.*, 1988) the changes are relatively low compared to those seen in cell lines and are not thought to be sufficiently large to account for the high degree of tolerance achieved *in vivo*. It must also be pointed out that other groups have reported either upregulation of receptor protein and mRNA (Brady *et al.*, 1989; Lewis *et al.*, 1984; Rothman *et al.*, 1989), or no change at all (Dum *et al.*, 1979; Hitzemann *et al.*, 1974). It is also evident that unlike what is typically seen in cell lines and isolated tissue, the concentration of the opioid antagonist naloxone required to precipitate withdrawal symptoms decreases as the level of tolerance increases (Wei *et al.*, 1973). Finally, it has been demonstrated that in brain slice preparations that the physiological changes which mark the development of morphine dependence *in vivo* are often not seen indicating that

intact neural circuitry is required (Andrade *et al.*, 1983; North & Williams, 1983). Taken together, these results strongly suggest that rather than being a phenomenon associated solely with the endogenous opioid system, the development of morphine tolerance and dependence requires the involvement of multiple neurotransmitter systems in the brain.

It has, therefore, been proposed that one consequence of chronic morphine exposure is the upregulation of other neurotransmitter systems that normally oppose the activity of the endogenous opioid system (reviewed by Schulteis & Koob, 1996). Under conditions of chronic morphine exposure the brain attempts to restore homeostasis by increasing the activity of these anti-opioid systems which contributes to the development of tolerance. Finally, it is proposed that the withdrawal symptoms precipitated by the blockade of the endogenous opioid system with naloxone in morphine dependent animals are partially due to the elevated levels of the anti-opioid systems.

Several neuromodulators have been implicated in contributing to morphine tolerance and dependence. N-methyl-D-aspartate (NMDA) antagonists as well as nitric oxide synthase inhibitors both inhibit the formation of tolerance and dependence, strongly suggesting the involvement of glutamatergic transmission (Higgins *et al.*, 1992; Kolesnikov *et al.*, 1993; Trujillo & Akil, 1991)

In addition, several neuropeptides have been classified as "anti-opioid" because of their ability to antagonize the effects of morphine and opioid peptides (reviewed by Rothman, 1992). It is proposed that these anti-opioid peptides may contribute significantly to the formation of tolerance and dependence. In support of this hypothesis, cholecystokinin (CCK) opposes opioid-mediated analgesia (Faris *et al.*, 1983; Zetler, 1980) and CCK receptor antagonists can prevent the development of morphine tolerance (Dourish *et al.*, 1990). In addition, neuropeptide FF (NPFF), initially described for its cardiovascular

activity, was later demonstrated to potently antagonize the analgesic effects of morphine and opioid peptides (Tang *et al.*, 1984a). Tang et al. also reported that NPFF immunoreactivity is elevated in the rat brain and spinal cord upon morphine exposure. In addition, NPFF antibodies have been used to reverse morphine tolerance (Lake *et al.*, 1991). Finally, intacerebroventricular (icv) injection of NPFF and intraperitoneal (ip) injection of NPFF analogs are reported to precipitate withdrawal symptoms in morphine dependent rats (Malin *et al.*, 1993a; Malin *et al.*, 1990b)l. Another peptide, Tyr-MIF-1, has also demonstrated anti-opioid properties with respect to opioid-mediated analgesia (Kastin *et al.*, 1985). In addition, this peptide appears to be upregulated during chronic morphine treatment (Zadina *et al.*, 1989) and precipitates withdrawal in dependent rats when delivered icv (Malin *et al.*, 1993b). Thus there is substantial evidence for the upregulation of anti-opioid peptides during chronic morphine treatment, as well as the involvement of these peptides in the development of tolerance and dependence.

Orphanin FQ, also called nociceptin because of its activity in analgesia assays (abbreviated here as OFQ), was originally identified as an endogenous ligand for the orphan opioid receptor-like receptor (abbreviated here as OFQR), variously refered to as ORL-1, LC132, XOR and KOR3 (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995). As is the case with members of the endogenous opioid peptides the mature seventeen amino acid OFQ peptide is proteolytically processed from a larger precursor called preproOFQ (ppOFQ). OFQ, in fact, shares considerable amino acid sequence homology to the classic opioid peptides, particularly with dynorphin (Mollereau *et al.*, 1996; Nothacker *et al.*, 1996). At the cellular level the OFQ receptor, similar to the classic opioid receptors, is G_i/G_o-coupled and inhibits adenylyl cyclase (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995), activates inwardly rectifying K^+ currents in various neuronal populations (Conner *et al.*, 1996; Vaughn & Christie, 1996; Vaughn *et al.*, 1997; Wagner *et al.*, 1998) and inhibits Ca^{++} currents (Abdulla & Smith, 1997; Connor *et al.*, 1996; Knoflach *et al.*,

1996) in response to ligand binding. Despite these similarities with the classic opioid receptors, OFQR does not bind any of the known opioid receptor agonists or antagonists with significant affinity (Bunzow *et al.*, 1994; Mollereau *et al.*, 1994). Likewise, the OFQ peptide does not bind with high affinity to any of the classic opioid receptors (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995).

The OFQ system has received considerable attention of late because of its ability to functionally antagonize the analgesia produced by morphine, opioid peptides, stress and electroacupuncture when injected icv (Mogil *et al.*, 1996b; Mogil *et al.*, 1996a; Tian *et al.*, 1997b). OFQ's functional anti-opioid supraspinal activity is in contrast to its activity in the spinal cord where it appears to potentiate the effects of morphine (Grisel *et al.*, 1996; Tian *et al.*, 1997a). Since OFQ acts as an anti-opioid with respect to supraspinal analgesia we asked whether the peptide might play some role in the development of tolerance and dependence. In support of our hypothesis there is dense expression of OFQ and OFQR mRNA in several brain regions that have been implicated in the development of dependence including the locus coereleus, amygdala, hypothalamus and the raphe nuclei of the brainstem (Darland & Grandy, 1998; Darland *et al.*, 1998; Houtani *et al.*, 1996; Maldonado *et al.*, 1992; Nothacker *et al.*, 1996). Therefore, we decided to determine whether levels of OFQ peptide, ppOFQ mRNA and/or OFQR mRNA change during the development of morphine tolerance and dependence, as has been reported for the other anti-opioid peptides described above. In addition, we also explored the possibility that exogenous OFQ can alter certain aspects of opiate dependence.

2. Results

Mice were made analgesically tolerant and physically dependent on morphine

C57BL/6J mice were chosen for these experiments because of their availability and because their responses to morphine are well-characterized (Belknap & O'Toole, 1991). We followed a paradigm of administering ip injections twice daily to render them morphine tolerant and dependent. To test for the development of tolerance the change in tail withdrawal latency from baseline after injection of either morphine or saline was measured (Ben-Bassar *et al.*, 1959). Figure 1 is representative of the tolerance to morphine that was developed, showing decreased tail withdrawal latency after the series of morphine injections. In contrast, there was no significant change in tail withdrawal latency for saline injected control mice over the course of the study. We next tested the animals for physical dependence on day ten by injecting cohorts of mice that had received either saline or morphine with 5 mg/kg naloxone ip and tabulating the withdrawal signs displayed during a five minute interval, five minutes post injection. By far the most prevalent withdrawal sign precipitated by naloxone in the morphine dependent mice was escape jumping with the animals displaying an average of 17.3 ± 3 jumps over the course of the trial period. In contrast, naloxone did not elicit any jumping in the saline-treated, control mice.

OFQ peptide levels increase in the hypothalamus, midbrain and brainstem of morphine-dependent animals

Extensive surveys using *in situ* hybridization in rat have demonstrated high expression of ppOFQ and OFQR mRNA in several brain areas thought to be involved in the development of morphine tolerance and dependence (reviewed by Koob *et al.*, 1992), including the locus coeruleus (LC) in the dorsal brainstem, raphe magnus (RM) in the ventral brainstem, medial thalamus (MT), frontal cortex (FCX), hypothalamus (HYP) and the amygdala (AMG) (reviewed by Darland & Grandy, 1998; Darland *et al.*, 1998). This

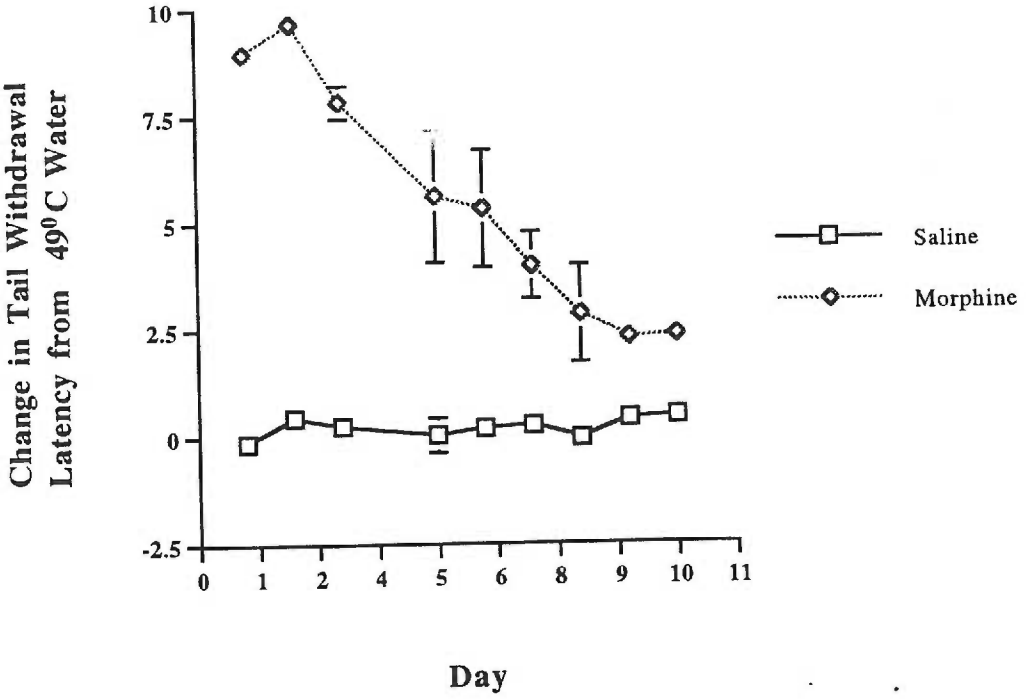

Figure 1. C57BL/6J mice were rendered tolerant to the analgesic effects of morphine. Animals were injected ip with 15 mg/kg morphine dissolved in saline or saline alone twice a day for 10 days. Each day the level of analgesia in the animals was determined by measuring the change in the time taken by the mice to withdrawal their tails from a 49°C water bath before and after injection. Tolerance in the morphine-treated animals (open diamonds) began to develop after day 2 as evidenced by the progressive decrease in tail withdrawal latency change. In contrast, saline treated controls (open squares) display almost no change in their tail flick latency over time.

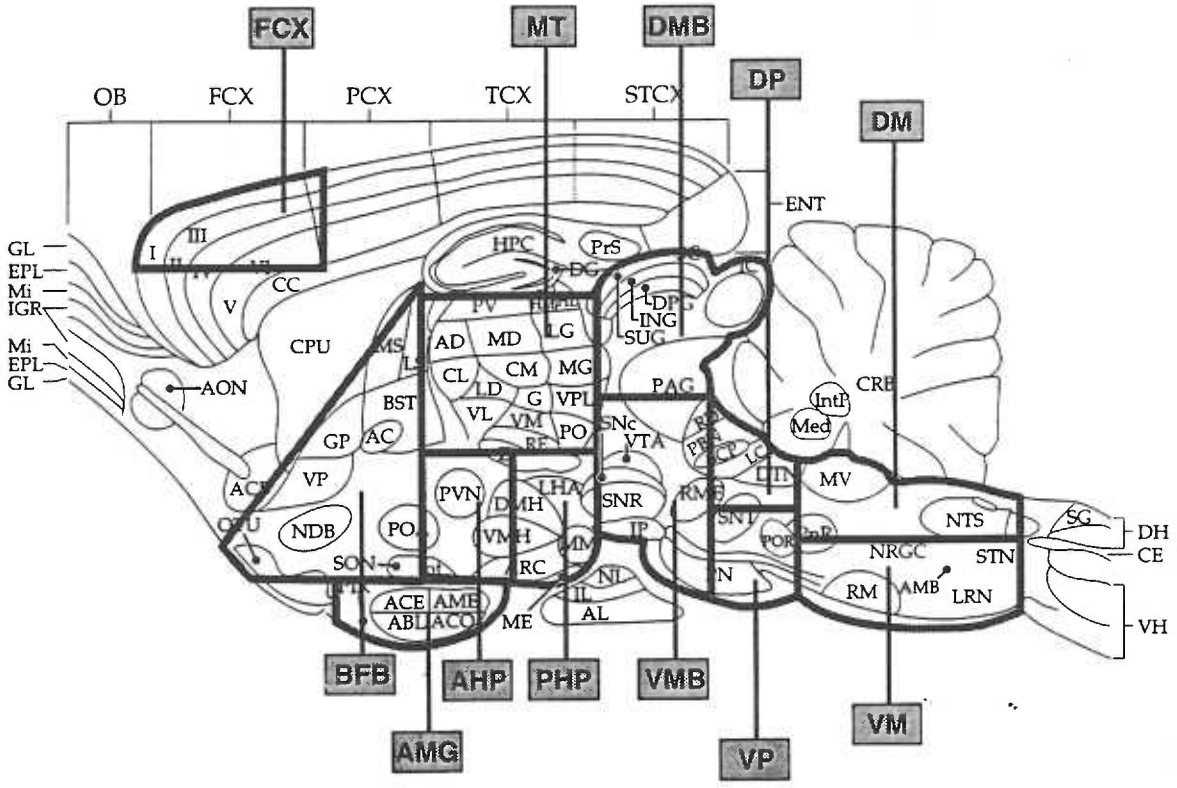

Figure 2. A schematic of the brain regions dissected. The dissections used for RNA isolation and analysis were somewhat different. The anterior and posterior hypothalamus tissues were pooled as were the dorsal and ventral portions of the midbrain and brainstorm. The sagittal section actually depicts an adult rat brain but serves the purpose of illustration (adapted from Mansour *et al.*, 1995). Abbreviations for the individual nuclei are listed in chapter 1, Figure 6. Mice that were morphine-tolerant and dependent were sacrificed one hour after their final injection of morphine, their brains removed and dissected as the figure shows. The abbreviations labeling the shaded areas appear in later figures and are as follows; AHP, anterior hypothalamus; AMG, amygdala; VFB, ventral forebrain; DM, dorsal medulla; DMB, dorsal midbrain; DP, dorsal pons; FCX, frontal cortex; MT, medial thalamus; PHP, posterior hypothalamus; VMB, ventral midbrain; VP ventral pons.

distribution is also found in the mouse (our unpublished observations and Houtani *et al.*, 1996; Nishi *et al.*, 1997). We therefore examined these areas for changes in expression of OFQ and its receptor with chronic morphine. In addition, the ventral forebrain (VFB) was examined because of its dense expression of precursor and receptor RNA and because portions of it have been implicated as being a neural substrate for some of the motivational signs of opiate withdrawal (Stinus *et al.*, 1990). Figure 2 schematically represents the brain dissection used in our studies that monitored OFQ peptide levels, as well as ppOFQ and OFQR mRNA levels in these experiments.

First we examined the aforementioned brain regions for changes in the level of OFQ peptide in morphine dependent and naive mice using a radioimmunoassay (RIA) (Quigley *et al.*, 1998). Due to the limited amount of tissue available, the LC and RM were not dissected individually but were combined with sections of the dorsal and ventral brainstem, respectively. Likewise, the VFB included tissue from the bed nucleus of the stria terminalis (BST), the preoptic area (POA), septum and ventral striatum. Of the areas tested in morphine-dependent animals the anterior hypothalamus (AHP), the ventral midbrain (VMB), ventral medulla (VM), and dorsal pontine area (DP) all displayed a significant elevation in OFQ peptide levels above saline-treated controls, increasing by 76%, 46%, 61% and 65%, respectively (Figure 3). The posterior hypothalamus (PHP), FCX, AMG, VFB, MT dorsal midbrain (DMB) and medulla (DM) of morphine dependent mice displayed no significant change in OFQ peptide from saline-treated animals.

We also evaluated the changes in OFQ peptide levels in mice after a single injection of saline or morphine using an RIA. Since the morphine-dependent and saline control animals were sacrificed 1 hour after receiving their final morphine injection it was possible that the increases in peptide levels we detected were a response to acute morphine exposure rather than a consequence of dependence. Since the largest change in OFQ peptide level was seen

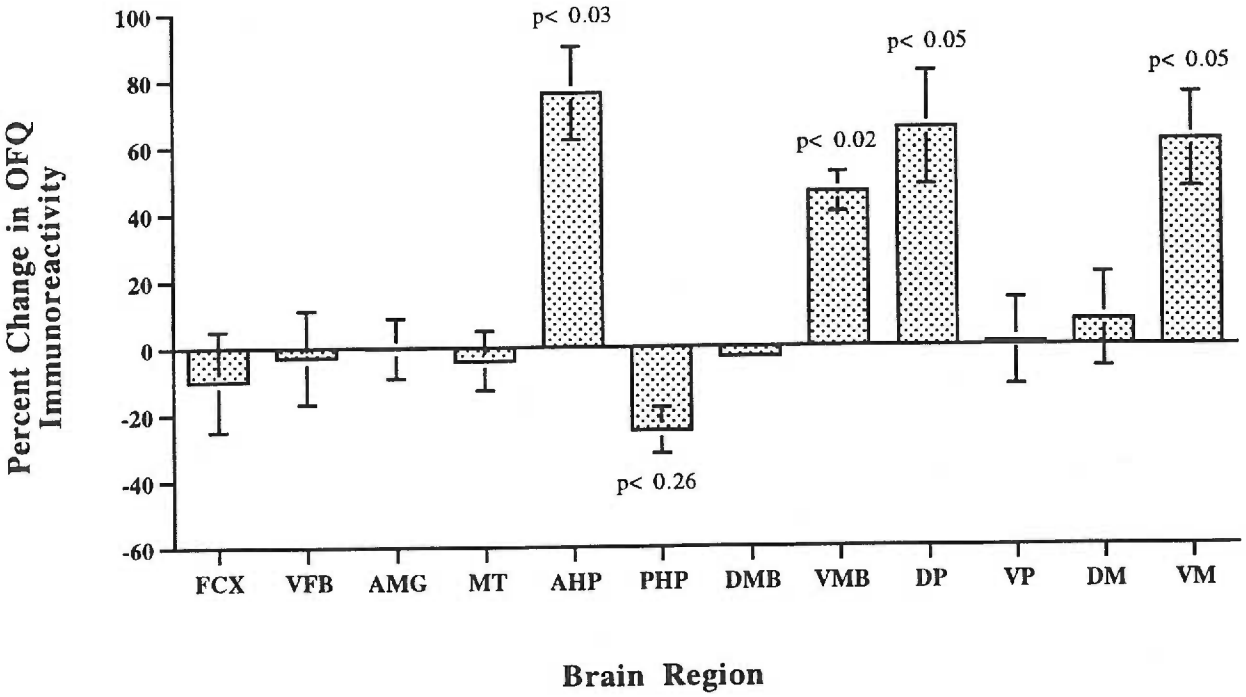

Figure 3. Changes in OFQ peptide immunoreactivity as measured by RIA in various brain regions of morphine-dependent mice. The changes are expressed as a percentage of the levels detected in morphine-naive controls. OFQ peptide levels were determined by the level immunoreactivity in protein lysates assayed by RIA. Significant increases ($p < 0.05$)in OFQ were detected in the anterior hypothalamus (AHP), the ventral midbrain (VMB), the dorsal pons (DP) and the ventral medulla (VM). In contrast, no significant changes in OFQ peptide were detected in any of the other tissues tested. The error bars represent ± SEM. The statistical significance was determined by using a one way ANOVA.

in the anterior hypothalamus, we tested this tissue in six previously drug-naive mice 1 hour after receiving a single injection of either saline or morphine (15 mg/kg). The hypothalamus displayed a slight increase in OFQ peptide of 16% which was not statistically significant ($p < 0.07$).

In opiate-tolerant mice ppOFQ mRNA levels increase in the basal forebrain, ventral medulla and decrease in amygdala

We next examined mRNA levels of both the OFQ precursor and receptor using RNase protection and *in situ* hybridization in order to determine whether the changes in peptide levels displayed with the development of morphine dependence coincided with increased mRNA synthesis. We analyzed the same brain areas described for peptide, however, because of the limited amount of tissue available for RNA extraction, the anterior and posterior hypothalamic tissues were combined (HYP in Figure 4), as were the dorsal and ventral portions of the midbrain and brainstem (DBS and VBS in Figure 4). Of the areas tested only the ventral forebrain (VFB) displayed a significant upregulation in ppOFQ mRNA with a 40% increase (Figure 4A). Interestingly, the amygdala (AMG) showed a 30% decrease in ppOFQ mRNA. We detected no change in the levels of ppOFQ for the FCX, MT, DBS, VBS, and HYP. We also detected no significant change in OFQR mRNA for any of the brain regions tested.

To complement the RNase protection studies, we performed an extensive survey for ppOFQ mRNA in morphine-dependent and control mouse brains using *in situ* hybridization. As was seen by RNase protection, there were few detectable differences between the two groups of animals. However, there were notable exceptions including the medial amygdala in which we detected a 20% decrease in ppOFQ mRNA and the ventral medulla which displayed a modest (16%), but statistically significant increase in the

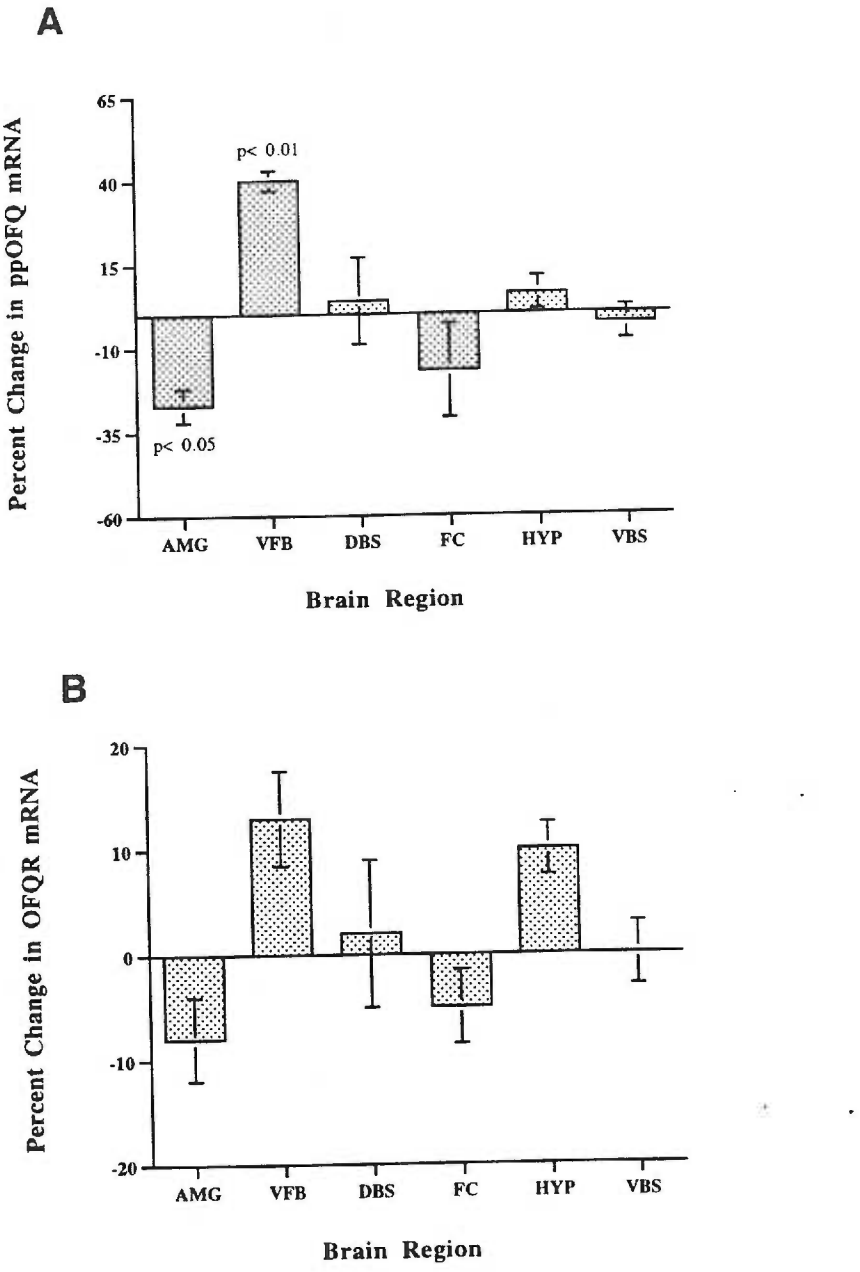

Figure 4. Changes in ppOFQ and OFQR mRNA levels as measured by RNase protection in various brain regions of morphine-dependent mice. Changes in ppOFQ and OFQR are expressed as a percentage of the levels detected in morphine-naive controls. RNA values for ppOFQ (A) and OFQR (B) were determined by densitometric scanning of RNase protection gels followed by normalization to cyclophilin, an internal control transcript. During the RNA isolation anterior and posterior hypothalamic tissue were combined (HYP) as were the dorsal and ventral portions of the midbrain and brainstem (DBS and VBS respectively). A significant increase in ppOFQ mRNA was detected in the ventral forebrain (VFB) and a significant decrease was seen in the amygdala (AMG). No changes in ppOFQ mRNA were seen in any of the other tissues tested. No significant changes in OFQ receptor mRNA were detected in any of the tissues tested. The error bars represent ± SEM.

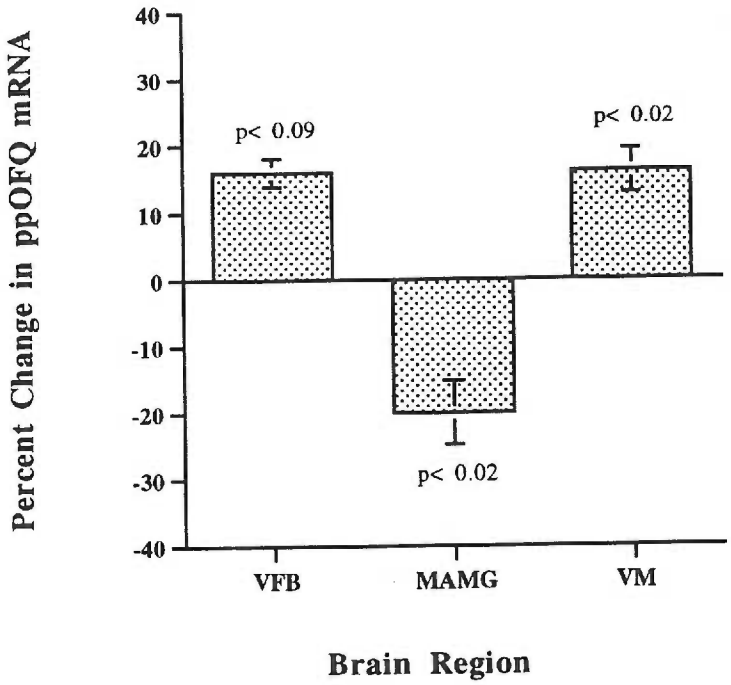

Figure 5. ppOFQ mRNA levels changed in morphine-dependent mice as measured by situ hybridization. Differences in ppOFQ expression between morphine-dependent and naive mice were determined by densitometrically analyzing the in situ autoradiographs of comparable brain sections . The values for the morphine-dependent animals are expressed as a percentage of their saline control counterparts. A significant increase (16%) in ppOFQ mRNA was detected in the ventral medulla (VM), consistent with expression in the raphe magnus. A significant decrease in ppOFQ was seen in the amygdala consistent with expression in the medial division (MAMG). The error bars represent ± SEM. The statistical significance was determined by using a one way ANOVA.

precursor (Figure 5). We also detected a small increase (16%) in ppOFQ mRNA in a ventral forebrain region which includes divisions of the BST, the substantia innominata and the POA. However, the difference proved to be statistically insignificant, in part due to differences in the quality of sectioning through this region and consequently should benefit from further study.

Intracerebroventricular injection of OFQ into morphine dependent mice

The elevated levels of OFQ immunoreactivity in several brain regions of morphine-dependent mice suggested to us the possibility that OFQ might mediate specific aspects of withdrawal. To investigate this possibility dependent and control mice were each given icv injections of OFQ. Animals were first lightly anesthetized with isoflurane and then injected with either 2.5 µl of artificial cerebrospinal fluid (ACSF) with or without 0.15 nmoles of OFQ peptide. Thus, there were four groups of animals: saline controls that received ACSF (S/ACSF) icv; morphine-dependent mice that received ACSF (M/ACSF) icv; saline controls that received OFQ (S/OFQ) icv; and morphine-dependent mice that received OFQ (M/OFQ) icv. The injected animals were placed individually into a cage with bedding and allowed 5 minutes to recover, by which time they were active and alert. For the next five minutes they were observed for physical withdrawal symptoms, total locomotion and exploratory rearing behavior.

Classic withdrawal symptoms such as jumping, writhing or wet-dog shaking were never observed. However, all members of both the saline control and morphine-dependent groups did display pilo erection and ptosis when injected with OFQ. Neither group displayed these symptoms when injected with ACSF alone. OFQ also decreased total locomotion, as measured by line crossing, in both control and morphine-dependent groups (Figure 6A). While the morphine-dependent animals that received OFQ appeared to move less on average than their saline control counterparts, the difference was not statistically

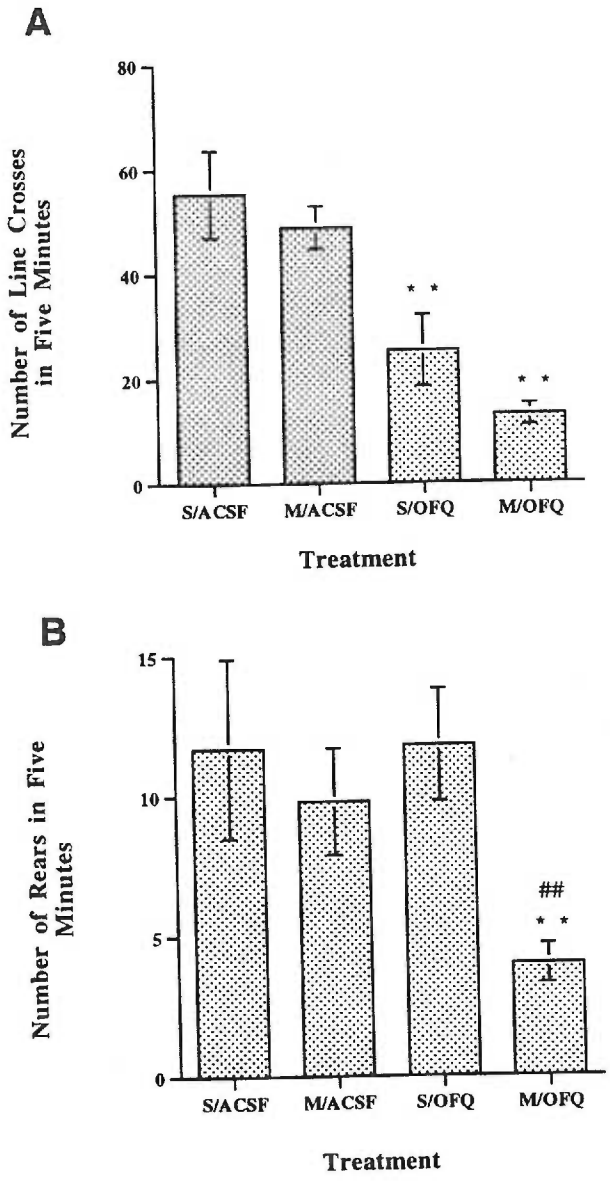

Figure 6. Total locomotion and rearing of morphine-dependent and naive mice following a 2 µl icv injection of artificial cerebrospinal fluid (ACSF) with or without 0.15 nmoles of OFQ peptide. (A) Locomotion was measured by recording the number of times the animals crossed the lines of a grid marked out in the experimental cage over a five minute interval five minutes after icv injection. (B)The number of rears observed during a five minute period five minutes after icv injection. Four groups of animals were tested: two groups that were morphine-dependent received either icv ACSF or ACSF with OFQ (M/ACSF and M/OFQ respectively), and two groups that were morphine-naive and received either icv ACSF or ACSF with OFQ (S/ACSF and S/OFQ). The error bars represent ± SEM. Statistical significance was determined by using a two way ANOVA. ## indicates a significant difference ($p < 0.01$) between the M/OFQ and S/OFQ groups. ** indicates a significant change ($p < 0.03$) of OFQ-treated animals from their respective ACSF-treated control counterparts.

significant ($p < 0.09$). Interestingly, OFQ dramatically reduced the number of exploratory rears in morphine dependent animals but not in saline controls (Figure 6B).

3. Discussion

We have characterized the levels of OFQ peptide as well as the mRNA encoding its precursor and receptor following chronic morphine exposure in order to evaluate the peptide's possible involvement in the development of tolerance and dependence. A twice daily injection procedure for 10 days was used to render C57BL/6J mice morphine tolerant and dependent. The decrease in the change of tail withdrawal latency after injection of morphine clearly demonstrated that the animals became tolerant to the drug's analgesic effects over time. Precipitation of withdrawal jumping behavior after injection with naloxone was also a clear indication that the animals were physically dependent.

Our results also demonstrated that the levels of OFQ peptide immunoreactivity and ppOFQ mRNA change during the development of tolerance and dependence. A dramatic rise in OFQ peptide, as measured by it immunoreactivity in an RIA, was displayed in the anterior hypothalamus and in certain areas of the midbrain and brainstem of dependent mice. The significant rise in peptide levels was not seen after a single acute dose of morphine and therefore the increases seen in dependent animals represents a neuroadaptive response to chronic morphine that would be consistent with anti-opioid activity of OFQ. Interestingly, with the exception of the ventral medulla, the increases in OFQ peptide observed in these areas did not coincide with an increase in ppOFQ mRNA, suggesting that they were due to increased release from the afferent projections originating in other brain regions. One possible afferent source of this OFQ peptide is the ventral forebrain which displayed a substantial elevation of ppOFQ/N mRNA levels and contains several nuclei which extend afferent projections to the hypothalamus, midbrain and brainstem (Conrad & Pfaff, 1973; Swanson & Cowan, 1979). Interestingly, the changes in OFQ peptide and

precursor mRNA levels we observed were not accompanied by detectable differences in receptor mRNA expression as determined by RNase protection.

Changes in the levels of other anti-opioid peptides in response to morphine have been described. Tang *et al.*, (1984a) reported a large increase (approximately 2 fold) in spinal NPFF immunoreactivity in the rat after acute infusion of morphine. Also, NPFF immunoreactivity has been reported to increase 60 and 150% in the hypothalamus and brainstem of morphine-dependent rats (Stinus *et al.*, 1995). Similar findings of increased peptide levels upon acute exposure to morphine have been described for CCK in the rat spinal cord (Tang *et al.*, 1984b). In addition, Zhou *et al.*. (1992) reported increases in CCK mRNA in whole rat brain coincident with the development of tolerance while others have reported mRNA increases in the amygdala (Pu *et al.*, 1994). Finally, Zadina *et al.*, (1989) reported that chronic morphine exposure decreased the number of Tyr-MIF-1 binding sites in the rat brain. It was argued that this decrease was most likely due to an elevation in the activity of the Tyr-MIF-1 peptide. In contrast to the increases detected in anti-opioid peptide and mRNA expression the expression of the classic opioid peptides displayed after chronic exposure to morphine markedly decreases (Romualdi *et al.*, 1992).

The increased levels of OFQ immunoreactivity seen in the anterior hypothalamus following chronic morphine administration suggests that the peptide may be involved in some of the neuroendocrine aspects of tolerance and dependence. The anterior hypothalamic tissue dissected in these studies should have included the paraventricular nucleus (PVN). The PVN is the major source of corticotrophin releasing hormone (CRH) that regulates the hypothalamic-pituitary-adrenal stress axis (HPA). The PVN receives extensive regulatory afferent projections from nuclei in the ventral forebrain including the BST, POA and substantia innominata (Conrad & Pfaff, 1973; Swanson & Cowan, 1979). That the OFQ receptor is densely expressed in the PVN (see Chapter1 Figure 2D), that the

OFQ peptide can elevate corticosterone levels in the blood (D.P. Devine et al. Neuroscience abstracts 23 part 2 #480.9) and has potent anxiolytic properties (Jenck *et al.*, 1997) suggests that OFQ may be an important regulator of the HPA. It is known that acute doses of morphine activate the HPA, as determined by elevated corticosterone levels in blood; tolerance to this response develops following chronic exposure to the drug; and naloxone-induced withdrawal is marked by large increases in plasma corticosterone (Ingar & Kuhn, 1990). Therefore, an intriguing hypothesis is that OFQ, perhaps originating from ventral forebrain afferent projections, modulates the changes in the HPA activity that are seen during the development of morphine tolerance and dependence.

Increased OFQ peptide and precursor mRNA in the ventral brainstem also has potentially important implications for the development of analgesic tolerance. The rostral ventral medulla (RVM) is an important relay point in the descending pathways that modify the transmission of painful stimuli at the level of the spinal cord (Fields *et al.*, 1991). Morphine injected into the RVM produces analgesia by disinhibiting the inhibitory neurons which project to the spinal cord (Heinricher *et al.*, 1994). Interestingly, OFQ opposes the analgesic effects of morphine mediated by RVM (Heinricher *et al.*, 1997). Increased OFQ in the RVM resulting from chronic morphine exposure may therefore contribute to the formation of analgesic tolerance.

Morphine-dependent animals also exhibited increased OFQ peptide, as determined by RIA, in the dorsal pons and ventral midbrain. While we were unable to locate the specific nuclei displaying these increases several intriguing possibilities exists. The ventral midbrain contains the ventral tegmental area (VTA) which expresses dense levels of OFQR mRNA and provides dopaminergic input to the nucleus accumbens. The nucleus accumbens has been implicated in the motivational aspects of withdrawal (Stinus *et al.*, 1990). Therefore, increased OFQ peptide levels in the VTA may impact the affective

aspects of morphine dependence. The dorsal pons tissue isolated during these dissections includes the locus coereleus (LC). The LC is the major noradrenergic nucleus in the brain, expresses high levels of OFQR mRNA (Chapter 1 Figure 4B), and has been implicated as a potent neural substrate for physical withdrawal (Maldonado *et al.*, 1992). While OFQ may not mediate physical withdrawal signs, it is possible that by regulating the output of this nucleus the peptide could affect the neuroendocrine and spinal analgesic aspects of morphine tolerance and dependence.

The dense expression of ppOFQ and its receptor in brain regions previously implicated as neural substrates for opiate withdrawal suggests to us a possible role for OFQ in the development of physical dependence. We have attempted to test this hypothesis by trying to precipitate withdrawal symptoms in morphine dependent mice with a single icv injection of OFQ peptide but we saw only mild signs of physical withdrawal such as pilo erection and ptosis. Other groups have precipitated more severe withdrawal symptoms including writhing and shaking using icv injection of NPFF and Tyr-MIF-1 (Malin *et al.*, 1990b; Malin *et al.*, 1993b). NPFF also precipitated withdrawal symptoms in opiate-naive animals creating what the authors referred to as a "quasi-abstinent" syndrome. It is possible that a similar situation exists for OFQ since icv injection of the peptide into morphine-naive mice resulted in a similar phenotype as observed in the dependent animals. On the other hand, the effects of icv OFQ injection may be unrelated to the dependent state of the animal.

Suppression of exploratory behavior, as measured by total locomotion, has been used as a behavioral index for the presence of affective or motivational withdrawal precipitated by naloxone in morphine dependent rats (Higgins & Sellers, 1994; Schulteis & Koob, 1996). Therefore, we measured total locomotion, as well as rearing behavior, to determine whether icv injection of OFQ into dependent mice could precipitate some the same motivational withdrawal symptoms. OFQ had a significant negative effect on total

locomotion compared to ACSF injected controls. However, while the morphine-dependent mice appeared to display less locomotion when injected with OFQ, the difference from their morphine-naive counterparts proved not to be statistically significant. OFQ had previously been reported to suppress locomotor activity in outbred strains of mice but not at the doses used in these experiments (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995).

A significant effect of OFQ icv injection into morphine-dependent mice was observed with respect to rearing. One interpretation of this decrease in rearing after injection could be a suppression of exploratory behavior, a symptom of motivational withdrawal. The changes in ppOFQ mRNA expression in the amygdala, a brain region implicated as a neural substrate for motivational withdrawal (Stinus *et al.*, 1990), supports this conclusion. However, other possibilities confound the analysis. Morphine-dependent mice injected with OFQ displayed behavior similar to the stereotypy resulting from high, acute doses of amphetamines and morphine, which consisted of intent gnawing on the bedding around them (Ernst, 1967; Randrup & Munkvad, 1967). Thus, the decrease in locomotion and rearing observed for morphine-dependent animals may not reflect precipitation of withdrawal but instead a nonspecific behavioral response that is exacerbated by morphine dependence. Therefore, at this time any conclusions regarding motivational aspects of withdrawal must be drawn with caution.

Currently, it is difficult to assess the role, if any, that OFQ plays in the development of morphine tolerance and dependence. The increased levels of peptide and mRNA displayed in response to chronic morphine and observations of delayed tolerance in OFQR knock-out mice (Ueda *et al.*, 1997) are consistent with the hypothesis that OFQ is important for the development of analgesic tolerance. We were unable to precipitate the extreme withdrawal signs observed after naloxone treatment. This is consistent with observations made in morphine-dependent rats by Tian *et al* . (1997a). It is possible that higher doses of OFQ,

on the order of 1-10 nm, may be required to precipitate classic withdrawal signs in mice. However, higher doses of OFQ, comparable to those used in the NPFF studies, produced severe ataxia which confounded the analysis. We did observe a decrease in what could be interpreted as exploratory behavior, a sign of motivational withdrawal, but again other behavioral considerations limit the interpretation of the data. To fully explore the possible role of OFQ in the development of morphine tolerance and dependence will require the use of antagonists which are only now becoming available (Guerrini *et al.*, 1998).

Discussion and Future Directions

1. Localization of ppOFQ and OFQR mRNA

Our survey of ppOFQ and OFQR mRNA expression using *in situ* hybridization reveals that both precursor and receptor distribution are widespread implying that the peptide is extremely diverse in its function. The OFQR mRNA expression patterns that we have detected are consistent with electrophysiological data reported by others which suggest that the peptide can regulate the activity of neurons in several brain regions including the cortex, hippocampus, VTA, dorsal raphe, PAG, LC, RVM, spinal cord as well as neurons in the suprachiasmatic, ventromedial and arcuate hypothalamic nuclei. Therefore, it is not surprising that the OFQ peptide has been reported to modulate several aspects of behavior and physiology including analgesia, anxiety, feeding, sexuality, audition, locomotion, learning and memory, reward, vasodilatation, water balance, gut motility, circadian rhythm, retinal and neuroendocrine function. Both in terms of distribution and function, OFQ's diversity matches that of the classic opioid peptides.

Given the similarities in structure, function and distribution to the endogenous opioids how does OFQ exert its functional anti-opioid effects? One explanation is that the endogenous opioids and OFQ utilize distinct components in the neural circuitry. A good example of this is the VTA, one of the main neural substrates governing reward in the brain. Morphine stimulates the activity of dopaminergic (DA) neurons in the VTA (Johnson & North, 1992) thereby increasing DA release in the nucleus accumbens (Devine *et al.*, 1993) and stimulating locomotor activity (DiChiara & Imperato, 1988a). OFQ has the opposite effect, hyperpolarizing DA neurons (A. Bonci unpublished. communication), suppressing the release of DA in the nucleus accumbens (Murphy *et al.*, 1996), and decreasing the locomotor effects of MOR agonists (P. Kalivis unpublished communication). Hence, the functional anti-opioid activity of OFQ is most likely a reflection of disparate expression patterns of OFQ, the opioids and their receptors within the VTA. MORs, which bind morphine with high affinity, are expressed on the inhibitory

GABAergic interneurons which regulate activity of the DA cells. in the VTA. Inhibition of these GABAergic cells, therefore, indirectly increases DA output from the VTA. OFQ could exert its anti-opioid effects on the reward circuits by binding to receptors expressed by the DA neurons themselves. Which cells in the VTA actually express OFQR remains to be determined.

Differences in the neural circuitry may also be responsible for OFQ's functional anti-opioid activity with respect to supraspinal analgesia. In addition to effects at the spinal cord level, morphine exerts a powerful influence on nociception through its effects in certain brain regions including the RVM. By activating inhibitory interneurons morphine activates descending projection neurons in the RVM (termed "off" cells) which in turn inhibit the transmission of noxious information entering the spinal cord from the periphery (Fields *et al.*, 1991; Heinricher *et al.*, 1994). OFQ blocks the effects of morphine on the RVM by inhibiting the activity of these same projection neurons (Heinricher *et al.*, 1997). Since one major effect of OFQ on neurons is to hyperpolarize them, the effects in the RVM may be mediated by OFQ receptors expressed by the "off" cells. Again this remains to be demonstrated.

The distribution of the OFQ immunoreactivity and ppOFQ mRNA is also similar in many respects to many of the other anti-opioid peptides. For example, high concentrations of NPFF are seen in the spinal cord, ventral brainstem and hypothalamus (Stinus *et al.*, 1995). CCK peptide is detected at high levels in the PAG and spinal cord while the amygdala, cortex, hippocampus, thalamus and VTA densely express CCK mRNA (Pu *et al.*, 1994). High concentrations of Tyr-MIF-1 peptide are also observed in the spinal cord as well as the hypothalamus (Reed *et al.*, 1994). High expression of these anti-opioid peptides, including OFQ, in the spinal cord is consistent with their role in modulating nociception. The same can be said for their expression in certain brain regions involved in

regulating the transmission of pain such as the ventral brainstem, PAG and amygdala. As with OFQ, these peptides can have anti-opioid effects unrelated to analgesia. CCK, for example, suppresses feeding and decreases dopamine release in the nucleus accumbens (reviewed by Crawley, 1991). How the OFQ system interacts with the other anti-opioid peptides is a question that warrants further investigation.

To more completely understand the interactions of OFQ with the endogenous opioids, anti-opioids, as well as other neurotransmitter systems a more thorough examination of ppOFQ and OFQR expression will have to be performed. In Chapter 1 we alluded to the possible interaction of OFQ with DA and other monoamine systems in the VTA-SNC, arcuate nucleus (ARC), LC and raphe nuclei. OFQ may also play a role in regulating GABA release in the reticular thalamus, BST and VTA. Other neurotransmitters may be regulated by OFQ including glutamate in the cortex and hippocampus and neuropeptide Y in the ARC and intergeniculate leaflet (IGL). Experiments combining tyrosine hydroxylase and glutamic acid decarboxylase immunohistochemistry with ppOFQ and OFQR in situ hybridization will help determine which cells synthesize the peptide and its receptor and whether the peptide acts pre- or post-synaptically in regulating other transmitters.

Combining retrograde labeling with immunohistochemistry and in situ hybridization will also aid in our understanding of OFQ circuitry. Do the efferents projecting from the BST to the brainstem and hypothalamus contain OFQ? Are some of the fibers projecting from the IGL to the SCN OFQ-positive? Where does the OFQ in the hypothalamus come from? Do projection neurons of the spinal cord and descending nociception modulatory pathways express OFQ? These are important questions that can be answered, in part, by future retrograde studies.

Finally, detailed mapping of OFQ peptide binding sites, such as those reported for the opioid receptors by Mansour et al (1995) will also aid our understanding of OFQ circuitry and may provide a means by which can be used to evaluate the dynamic changes in OFQ activity during the development of morphine tolerance and dependence.

2. Changes in OFQ immunoreactivity and ppOFQ mRNA levels with the development of dependence and analgesic tolerance

We have shown that the levels of OFQ immunoreactivity as well as ppOFQ mRNA display significant changes that coincide with the development of morphine tolerance and dependence. Peptide levels were found to be increased in the anterior hypothalamus, dorsal pons, ventral midbrain and medulla of morphine-tolerant and dependent animals. PreproOFQ mRNA increased in the ventral forebrain and decreased in the amygdala of morphine-dependent mice. A summary schematic depicting the possible OFQ circuitry upregulated during the development of morphine tolerance and dependence is shown in Figure 1. Thus, OFQ peptide regulation during chronic morphine exposure appears to be similar to that of the other anti-opioids, consistent with their involvement in the development of tolerance and dependence (Pu *et al.*, 1994; Stinus *et al.*, 1995; Zhou *et al.*, 1992).

A role for OFQ in the development of analgesic tolerance is also suggested by experiments which demonstrated a delay in morphine tolerance in transgenic mice which lack the OFQ receptor (Ueda *et al.*, 1997). One explanation for this partial effect on analgesic tolerance observed in the OFQR-deficient mice is the choice of background strain. C57BL/6J mice do not develop the degree of analgesic tolerance that some other strains do. It would be interesting to evaluate the loss of tolerance in OFQR-deficient transgenic mouse bred in another background strain such as DBA/2J in which tolerance is more robust (Belknap & O'Toole, 1991). Another explanation for the partial effect is that several

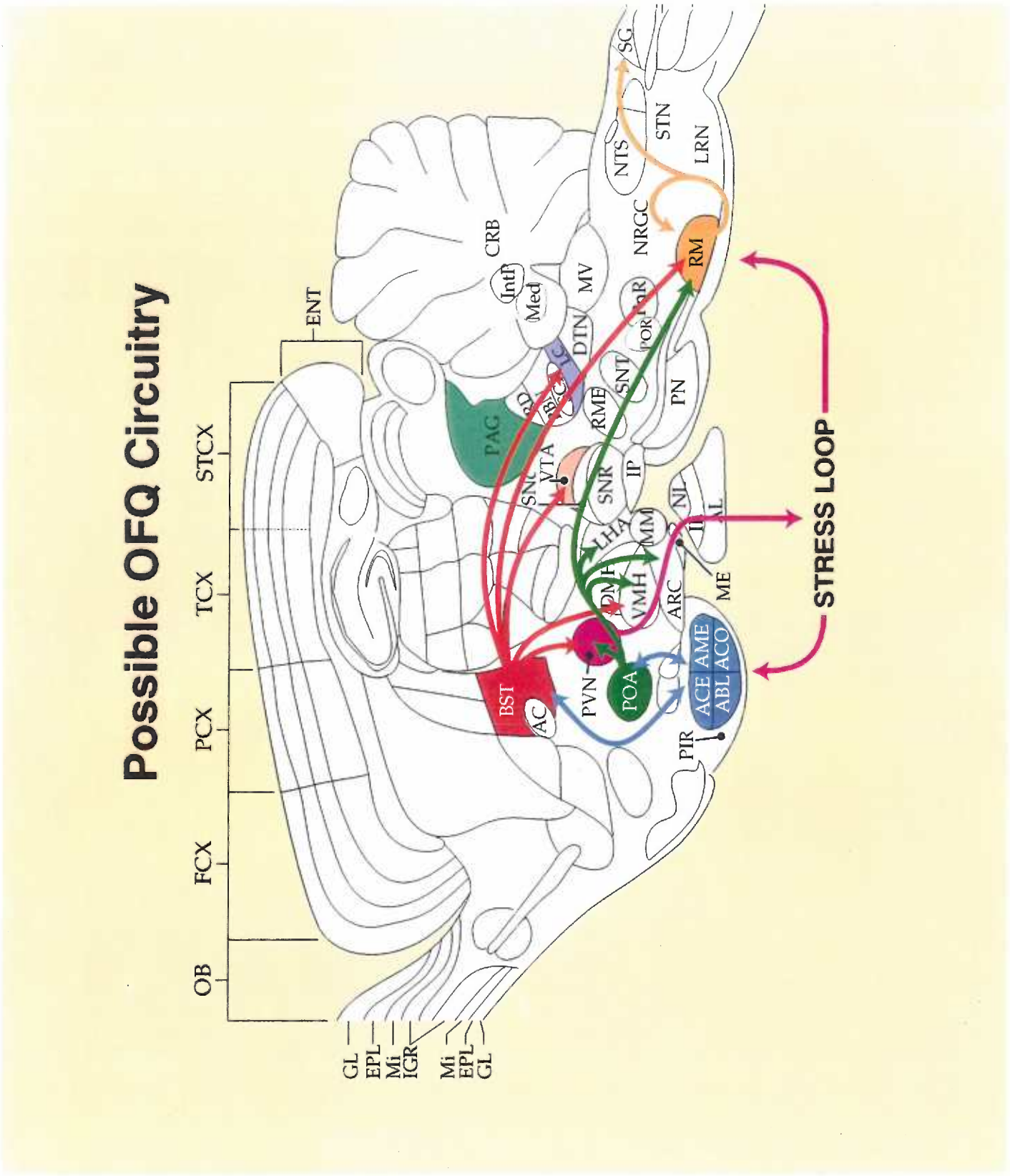
Possible OFQ Circuitry
OB
FCX
PCX
TCX
STCX
ENT
GL
EPL
Mi
IGR
Mi
EPL
GL
CRB
IntF
Med
MV
DTN
PAG
VTA
SNR
RME
SNT
PN
IP
LHA
MM
DMH
VMH
ARC
ME
NL
NRGC
NTS
SG
STN
LRN
RM
BST
AC
PVN
POA
ACE
ABL
AME
ACO
PIR
STRESS LOOP

Figure 1

neuromodulatory systems in several regions in the CNS contribute to morphine tolerance. The effects of morphine in the spinal cord and different brain regions act synergistically to produce analgesia (Bodnar *et al.*, 1991; Yeung & Rudy, 1980) and perhaps the same is true for opiate tolerance. OFQ may exert effects on analgesic tolerance in only a few of these regions. Also, The development of mouse strains that can be spatially and temporally rendered OFQR-deficient should permit examination of OFQ activity in discrete brain regions.

In our studies we chose to experiment with C57BL/6J mice primarily because of their well-characterized withdrawal response to morphine (Belknap & O'Toole, 1991). While C57BL/6J mice do not develop morphine tolerance as rapidly or to as great a degree as do some other strains, we were able to show that a significant degree of tolerance was achieved by twice daily injections over a ten day period. It would be interesting to compare the OFQ peptide levels we have demonstrated in C57BL/6J with those of other strains which differ in their behavioral response to morphine. For example, it might be expected that OFQ levels are higher in mouse strains which display higher degrees of analgesic tolerance to chronic morphine.

How might OFQ contribute to the development of opiate tolerance and dependence? Tolerance to the analgesic effects of morphine is accompanied by decreased activity of dorsal horn neurons that appears due to both the desensitization of opiate receptors as well as an activation of neural systems involving anti-opioid peptides (reviewed by (Johnson & Fleming, 1989)). Several reports indicate that in the spinal cord OFQ potentiates analgesia, decreases activity of dorsal horn neurons and lowers synaptic transmission from the periphery (Erb *et al.*, 1997; Grisel *et al.*, 1996; King *et al.*, 1997; Lai *et al.*, 1997; Liebel *et al.*, 1997; Tian *et al.*, 1997a; Wagner *et al.*, 1998; Wang *et al.*, 1996; Xu *et al.*, 1997)

although at this time there is not a consensus on these points (Dawson-Basoa & Gintzler, 1997; Stanfa *et al.*, 1996; Yamamoto *et al.*, 1997). The use of animals of different species, strains, sexes and behavioral states may be one source of discrepancy in these studies. An inhibitory effect of OFQ in the spinal cord, similar to the effect produced by morphine, suggests that the peptide does not have functional anti-opioid activity in the dorsal horn and therefore may not contribute to the spinally mediated aspects of tolerance. Of course this does not discount the possibility that OFQ may modulate the release of other anti-opioid peptides in the spinal cord which do contribute to tolerance.

It is also possible that OFQ may influence the development of analgesic tolerance by acting at supraspinal sites that are involved in nociceptive processing. The inhibitory actions of OFQ on the RVM projection neurons suggests the possibility that upregulation of the peptide during chronic morphine exposure might be a compensatory mechanism relevant to the development of tolerance. OFQ may exert a similar effect in the ventral PAG where the peptide attenuates morphine-induced analgesia (Morgan *et al.*, 1997), although it has not yet been determined where in the ventral midbrain OFQ peptide levels increase. Despite their well defined role in modulating nociceptive processing, it is not known if neurons of the RVM and PAG actually display morphine tolerance or if they contribute to the development of tolerance in the spinal cord.

Another possible supraspinal substrate of tolerance which could be regulated by OFQ is the locus coereleus (LC). We show an increase in OFQ immunoreactivity in the dorsal pons, an area which includes the LC. In addition to its many projections to other brain regions, the LC provides noradrenergic input to the spinal cord which appears to play a role in nociception (Korf *et al.*, 1974). Acute morphine inhibits the activity of LC neurons while tolerance to this inhibition develops after chronic exposure (Aghajanian, 1978). OFQR mRNA is expressed at high levels in the LC (Chapter 1 Figure 4B) and OFQ peptide

inhibits the activity of LC neurons (Conner *et al.*, 1996). While OFQ has the same effects on LC neurons as morphine, the peptide might exert functional anti-opioid effects on the output of the nucleus depending on which neurons it inhibits.

The LC, PAG and nuclei within the RVM, such as the raphe magnus, are all potent neural substrates for precipitating withdrawal symptoms in dependent rats (Maldonado *et al.*, 1992). While the question of whether OFQ can precipitate withdrawal remains open (see below), the fact that the peptide in potently regulates the output of these regions implies of some role for OFQ in morphine dependence. Yet another brain region implicated in morphine dependence and also rich in OFQ immunoreactivity is the hypothalamus.

In morphine-dependent mice the largest increase in OFQ peptide that we detected was in the anterior hypothalamus. While it is not yet certain where in the anterior hypothalamus the peptide increases occurred, one likely area is the PVN. As described in Chapter 2, this area is particularly important for regulating the HPA-stress axis. Although the mechanism is not yet clear OFQ has been reported to increase corticosterone levels in the blood which is consistent with its anxiolytic properties (Jenck *et al.*, 1997). The changes in OFQ levels we have detected may reflect the altered neuroendocrine responses of the HPA reported to occur during chronic morphine exposure. While corticosterone is believed to contribute to alcohol withdrawal seizures (Roberts *et al.*, 1994), its role in opiate dependence is unclear.

The HPA axis has also been implicated in other aspects of opiate addiction not directly related to dependence. High plasma corticosterone levels have been correlated with increased locomotor responses to a novel environment in certain inbred rat strains (Piazza *et al.*, 1993). Increased responsiveness and corticosterone levels have also been correlated with increased self-administration of morphine and other drugs of abuse (Piazza & LeMoal, 1996). Given OFQ's possible importance in regulating the HPA, as well as its potential

function in mediating reward, it would interesting to determine how hypothalamic levels of OFQ correlate with responsiveness in these rats.

In addition to interacting with other neurotransmitter and neuroendocine systems, OFQ may influence the development of morphine tolerance and dependence by interacting with neurotrophic factors. If the 1997 Neuroscience meeting was any indication, there is a growing interest in the role of neurotrophic factors in behavior, including drug addiction. At this meeting one group of investigators reported an upregulation of brain-derived growth factor mRNA in the LC during opiate withdrawal and suggested that neurotrophins might be involved in long term changes which occur in the LC following opiate exposure (Neuroscience Abstracts Vol. 23 part 1 # 27.14). Upregulation of neurotrophic factors in the spinal cord after chronic morphine exposure have also been reported (Hendry *et al.*, 1987). This is perhaps particularly relevant to OFQ given that the expression of the ppOFQ stimulates neurite outgrowth in the NS20Y neuroblastoma cell line (Saito *et al.*, 1996). The mechanism of this activity is unknown at present. Saito et al have also reported that preproOFQ is also expressed in areas of neuronal differentiation (Saito *et al.*, 1997). It would be interesting to see if ppOFQ affects the expression of the classic neurotrophic factors in these cells, in the embryo, and during the development of tolerance and dependence.

While the increases in OFQ peptide and its precursor mRNA certainly suggest that this system is involved in the development of morphine tolerance and dependence, it is only correlative evidence. In examining the anti-opioid properties of other peptides such as dynorphin, NPFF and CCK investigators have been able to draw on a wide range of pharmacological agents, including selective antagonists. To test some of the hypotheses described above better tools, including transgenic mice lacking the OFQ receptor and precursor, as well as selective antagonists will be necessary. Recently, a specific OFQR

antagonist has been reported (Guerrini *et al.*, 1998). With this compound it may be possible to test whether blocking OFQ activity is sufficient to prevent the formation of morphine tolerance and/or dependence. The antagonist could also potentially be used to characterize electrophysiological differences in the spinal cord and specific brain regions, including the RVM, PAG and the LC which occur following chronic morphine exposure.

3. Can OFQ precipitate withdrawal?

We attempted to precipitate withdrawal in morphine-dependent mice by administering OFQ icv (Chapter 2). In these experiments we detected mild signs of physical withdrawal, including pilo erection and ptosis in both morphine-dependent and naive animals injected with OFQ. After icv injection, all OFQ-treated mice displayed decreased locomotion, however there was a trend towards greater suppression in morphine-dependent animals. We also observed a decrease in rearing by morphine-dependent animals treated with OFQ.

In contrast to the results observed after icv injection of OFQ, NPFF and Tyr-MIF-1 precipitated symptoms in morphine dependent rats similar to the withdrawal syndrome produced by naloxone. Writhing was the most common sign and no jumping was reported in these studies. The difference in OFQ's activity may have several explanations. The most obvious explanation is that OFQ does not mediate the physical aspects of morphine dependence. This would be consistent with the results reported by Tian et al in morphine-dependent rats (Tian *et al.*, 1997a). However, one major difficulty in interpreting these results involves the route of administration. The injection of OFQ icv may generate nonspecific effects which interfere with the manifestation of withdrawal symptoms. A good example, is the effect OFQ had at higher concentrations on locomotion. Doses on the order of 1-10 nmoles may be required to produce withdrawal symptoms but the ataxia produced at that dose prevents analysis. Other means of manipulating the OFQ levels, such as stereotaxic injection, will be required to fully address whether the peptide precipitates

withdrawal. Transgenic mouse models might also be useful in this regard. The effects on the formation of morphine dependence in OFQR knock-out mice has not been reported.

The decreases in locomotion and rearing that were observed after icv injection of OFQ in morphine-dependent mice suggests the possibility that OFQ may mediate some aspects of affective, or motivational withdrawal. Suppression of exploratory behavior in rodents is one of the indices used to model the emotional state of addicts withdrawing from opiates (Higgins & Sellers, 1994; Schulteis *et al.*, 1994). OFQ clearly decreased locomotion in both the morphine-dependent and drug naive animals. although there was a trend towards increased suppression in the latter. The locomotor effect seen at lower doses was not due to ataxia since the animals readily and easily moved when prodded. However, we cannot dismiss the possibility that OFQ still had general locomotor effects which are somehow exaggerated in animals that have been exposed to chronic morphine.

Another interpretation of the decreased locomotion in drug-naive animals is that icv injection of OFQ produced a "quasi-dependent" state in a similar manner to the effect of NPFF on physical withdrawal in naive animals (Malin *et al.*, 1990b). The trend towards increased suppression of locomotion in morphine-dependent animals may then reflect true motivational withdrawal. The morphine-dependent animals displayed a decrease in rearing behavior. This could also be interpreted as suppressed exploratory behavior and therefore a sign of motivational withdrawal. However, alternative explanations can account for the decrease in rearing. OFQ appeared to stimulate oral behaviors similar to the stereotypies that results from exposure to high doses of amphetamine and morphine (Ernst, 1967; Randrup & Munkvad, 1967). Increases in striatal dopamine and serotonin, particularly in the caudate putamen and nucleus accumbens, appear to mediate this behavior (Ernst, 1972; Segal & Kuczenski, 1997). This behavior, which consisted mainly of continuous, intent gnawing on the bedding as well as excessive licking and grooming, was in OFQ-treated,

opiate-naive mice but more prevalent and exaggerated in the morphine-dependent animals. Since OFQ has inhibitory effects on monoaminergic neurons it is not obvious how the peptide stimulates this behavior. Again, as with the locomotor results, this effect of OFQ may not be specific to morphine-dependence but may be exacerbated by it.

Given the behavioral confounds encountered in our attempts to precipitate withdrawal it is difficult to conclude what role OFQ plays in the development of morphine dependence. Perhaps a better behavioral test to use in approaching this question would be conditioned place aversion. This test been used to measure affective withdrawal in rats (Higgins & Sellers, 1994; Mucha, 1987; Schulteis *et al.*, 1994) and may be particularly useful because OFQ does not appear to have aversive properties in drug naive animals (Devine *et al.*, 1996b).

Summary and Conclusions

The expression patterns exhibited by ppOFQ and its receptor suggest that this is an extremely important neuromodulatory system that is involved in many processes including nociception. In this respect OFQ is interesting because in some brain regions involved in nociception the peptide behaves as an anti-opioid. Our data show that chronic morphine exposure results in increased levels of OFQ peptide in many brain regions which may be important in the formation of tolerance and dependence. Therefore it is entirely likely that the peptide contributes to these phenomena. We were unable to demonstrate any relationship between OFQ and the physical aspects of morphine dependence, although given the experimental confounds encountered during this work we must be cautious about this conclusion. We do present evidence that OFQ may mediate some of the affective aspects of dependence although this awaits further testing. The future of this research is extremely promising as important experimental reagents such as transgenic mice and pharmacological antagonists are being developed. It is my hope that my research efforts

have identified a few avenues worthy of further study. As additional tools become available, these avenues can then be explored to expand our knowledge of this very important molecule.

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