Physiological and Pharmacological Characterization of the Nociceptin/Orphanin FQ Neuropeptide and the Nociceptin/Orphanin FQ Receptor

By

Denise I. Quigley

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Oregon Health Sciences University

CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. thesis of

Denise I. Quigley

has been approved

Professor in charge of thesis

Member

Member

Member

Member

Associate Dean for Graduate Studies

For my father, Jack Quigley, my strongest supporter
and greatest source of encouragement.

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Abstract

The nociceptin/orphanin FQ receptor (NOR) was originally identified based on its sequence similarity with opioid receptors. The endogenous peptide ligand nociceptin/orphanin FQ (N/OFQ) activates NOR and shares sequence homology with the classical opioid peptides. However, the dissimilarities between this receptor-ligand system and the opioid system may prove to be more interesting than the similarities. N/OFQ appears to possess anti-opioid properties and be involved in the modulation of feeding behavior, locomotion, cardiovascular function, spatial learning and stress. I have conducted a series of studies in an effort to begin to characterize the physiological and pharmacological properties of N/OFQ and NOR. First, I developed a radioimmunoassay (RIA) for N/OFQ that can detect the peptide in central tissues. The results of N/OFQ RIA studies and the anatomical distribution of NOR mRNA led us to examine the involvement of N/OFQ in stress and the circadian cycle. The N/OFQ RIA was a useful tool for measuring quantitative changes in N/OFQ levels in response to stress paradigms and is expected to be a useful tool for others studying the N/OFQ peptide. Observed changes in N/OFQ levels in these physiological studies lead us to examine how N/OFQ interacts with NOR to mediate physiological functions. I created mutant forms of NOR by site-directed mutagenesis to examine the molecular pharmacology of NOR and to determine specific NOR amino acid residues involved in N/OFQ binding and receptor activation. In the process of establishing a binding assay for the characterization of these NOR mutants, the intrinsic stability of N/OFQ was evaluated. I determined that N/OFQ is particularly sensitive to enzymatic degradation and identified putative degradation products via reversed phase-high performance liquid chromatography (RP-HPLC). The results of these studies reveal the importance of monitoring peptide integrity for reliable experimental results and will serve as a cautionary note to others in the NOR-N/OFQ field. Finally, with the establishment of a reproducible binding protocol, I examined N/OFQ interactions at NOR.

These studies suggested that charged NOR amino acid residues are important for N/OFQ binding and activation. Collectively, these data may provide valuable insight to the various functions of the N/OFQ-NOR system and establish some groundwork for further characterization of NOR-N/OFQ interactions.

Introduction

Pharmacology and Physiology of the Nociceptin/Orphanin FQ System

Pharmacology and Physiology of the Nociceptin/Orphanin FQ System

Denise I. Quigley[a] and David K. Grandy*

[a]Department of Molecular and Medical Genetics, Department of Physiology and Pharmacology, Oregon Health Sciences University, 3181 SW Sam Jackson Park Road, Portland, OR 97201

Corresponding Author:
David K. Grandy, Ph.D.
Department of Physiology and Pharmacology L-334
Oregon Health Sciences University
3181 SW Sam Jackson Park Road
Portland, OR 97201
phone: (503) 494-4671
FAX: (503) 494-4352
E-mail: grandyd@OHSU.edu

Abstract

The recently discovered nociceptin/orphanin FQ receptor (NOR) and its endogenous peptide ligand nociceptin/orphanin FQ (N/OFQ) have attracted much attention because of their similarities and, perhaps more importantly, their differences compared with opioid receptors and peptides. The N/OFQ peptide has been implicated in analgesia, hyperalgesia and the reversal of opioid mediated analgesia. The interpretation of *in vivo* and *in vitro* pharmacological studies has been confounded by the susceptibility of N/OFQ to degradation and enzymatic cleavage and the potential functionality of these resulting breakdown products. Lack of a selective NOR antagonist has further confounded the pharmacological and physiological characterization of this system. Mutagenesis studies were initiated in an effort to determine characteristics of NOR and N/OFQ that are essential for binding interactions and receptor activation. This introduction surveys recent pharmacological studies of the N/OFQ system and underscores the importance of standardizing pharmacological agents and protocols.

Key Words: nociceptin, orphanin FQ, nociceptin/orphanin FQ receptor (NOR), pharmacology, mutagenesis

I. Introduction

Three major types of opioid receptors have been described. These receptor subtypes, denoted as μ, δ and κ, were originally distinguished by their distinct pharmacological profiles and anatomical distributions. The cDNAs that encode each of these three receptor subtypes have been cloned and their deduced amino acid sequences reveal highly homologous proteins that are organized into seven putative transmembrane spanning domains. In spite of their distinct pharmacology, ligand binding to all three opioid receptor subtypes is antagonized by the compound naloxone. The opioid receptors couple to pertussis toxin-sensitive G proteins that mediate the inhibition of adenylyl cyclase and voltage-gated Ca^{2+} channels and activate an inwardly rectifying K^+ conductance (for review, see Knapp *et al.*, 1995; Reisine, 1995; Lambert, 1995; Standifer *et al.*, 1997; Satoh *et al.*, 1995). In addition to the extensive sequence homology shared by the opioid receptors, all of the endogenous opioid peptides share similar amino acid sequences (Fig.1). Opioid peptides share the canonical amino terminal sequence Y-G-G-F, a hallmark characteristic of this class of peptides. Moreover, all opioid peptides are derived from larger precursor proteins by prohormone convertase mediated cleavages at dibasic amino acid cleavage sites.

Given the clinical usefulness of morphine as a potent analgesic, an intense search was conducted for novel opioid-related drugs and/or receptor types that would not produce the unwanted side effects associated with chronic morphine use. Perhaps the most exciting recent discovery in the opioid field is the novel opioid-like receptor NOR (nociceptin/orphanin FQ receptor) (Mollereau *et al.*, 1994; Bunzow *et al.*, 1994; Wang *et al.*, 1994; Wick *et al.*, 1994; Fukuda *et al.*, 1994; Pan *et al.*, 1994) and its endogenous ligand, N/OFQ (nociceptin/orphanin FQ) (Reinscheid *et al.*, 1995; Meunier *et al.*, 1995).

Figure 1

N/OFQ	Phe-**Gly-Gly-Phe**-Thr-Gly-Ala-Arg-**Lys-Ser**-Ala-Arg-**Lys-Leu**-Ala-**Asn-Gln**
Dynorphin A	Tyr-**Gly-Gly-Phe**-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-**Lys**-Trp-Asp-**Asn-Gln**
α-endorphin	Tyr-**Gly-Gly-Phe**-Met-Thr-Ser-Glu-**Lys-Ser**-Gln-Thr-Pro-**Leu**-Val-Thr
Met-enkephalin	Tyr-**Gly-Gly-Phe**-Met
Endomorphin 1	Tyr-Pro-Trp-**Phe**-NH_2
Endomorphin 2	Tyr-Pro-Phe-**Phe**-NH_2

Figure 1. Comparison of N/OFQ amino acid sequence with opioid peptides. Amino acid residues that are common among N/OFQ and opioid peptides are highlighted.

Depending on the dose and site of administration, N/OFQ can produce analgesia (Rossi *et al.*, 1996; Yamamoto *et al.*, 1997; Erb *et al.*, 1997) act as an anti-opioid peptide (Grisel *et al.*, 1996; Mogil *et al.*, 1996b; Heinricher *et al.*, 1997) suppressing opioid-mediated analgesia (Zhu *et al.*, 1997). In addition, this system appears to impair motor function (Devine *et al.*, 1996), suppress spatial learning (Sandin *et al.*, 1997), influence feeding behavior (Pomonis *et al.*, 1996; Stratford *et al.*, 1997) and may play a role in various stress responses (Jenck *et al.*, 1997; Griebel *et al.*, 1999). The purpose of this review is to examine the pharmacology of the N/OFQ peptide-receptor system and affects of NOR or N/OFQ mutagenesis on the pharmacology and physiology of this system.

II. The Orphan Opioid Receptor and Its Endogenous Ligand

The cloning of the δ-opioid receptor in 1992 (Evans *et al.*, 1993; Kieffer *et al.*, 1993) led to the cloning of subsequent opioid and opioid related receptors. Using oligonucleotide primers based on the δ-opioid receptor sequence, RT-PCR and cDNA library screening led to the cloning of the μ- (Chen *et al.*, 1993) and κ- opioid receptors (Yasuda *et al.*, 1993) in 1993. In addition, several groups identified a cDNA that coded for a related G protein-coupled receptor, which was variously named LC132 (rat) (Bunzow *et al.*, 1994), ORL1 (human) (Mollereau *et al.*, 1994), KOR-3 (mouse) (Pan *et al.*, 1994), XOR-1 (mouse) (Wang *et al.*, 1994), Hyp 8-1 (mouse) (Wick *et al.*, 1994) and ROR-C (mouse) (Fukuda *et al.*, 1994). The receptor has been named NOR (nociceptin/orphanin FQ receptor) for its ligand and in keeping with the convention of the naming of the μ-, δ- and κ- opioid receptors (MOR, DOR and KOR, respectively).

The genes encoding the MOR, DOR, KOR and NOR share organizational similarity. The coding regions of these receptors contain two introns, one in the first intracellular loop and the second in the second extracellular loop (Mollereau *et al.*, 1994;

Nishi *et al.*, 1994). Similarities in their gene organization suggests that the NOR and the classical opioid receptors have a common evolutionary origin. At the amino acid level, the NOR is about 65% homologous (Chen *et al.*, 1994) and 50% identical (Mollereau *et al.*, 1994; Bunzow *et al.*, 1994; Wang *et al.*, 1994) to the classical opioid receptors. The putative second, third and seventh transmembrane domains of NOR are the most conserved, being approximately 80% homologous (Mollereau *et al.*, 1994; Chen *et al.*, 1994) and 65% identical to MOR, DOR and KOR (Mollereau *et al.*, 1994; Bunzow *et al.*, 1994; Lachowicz *et al.*, 1995). The NOR amino acid sequence is most divergent in its N- and C- termini and the second and third extracellular loops (Mollereau *et al.*, 1994; Lachowicz *et al.*, 1995). Overall, NOR is equally similar to each of the three classical opioid receptor types; however, the acidic second extracellular loop, perhaps most critical in binding specificity, most closely resembles that of KOR (Lachowicz *et al.*, 1995). In addition to the amino acid similarity among the transmembrane domains, the first, second and third putative intracellular loops, including the DRYXXV(I)XXPL motif in intracellular loop II, are highly conserved among NOR and the classical opioid receptors (Bunzow *et al.*, 1994; Wick *et al.*, 1994). These regions are thought to be involved in G protein-receptor coupling which initially suggested that NOR might couple to the same second messenger pathways as MOR, DOR and KOR (Bunzow *et al.*, 1994; Wick *et al.*, 1994).

The distribution of NOR has been examined at both the level of mRNA and protein. Among the areas in which NOR message expression is the highest are the hypothalamus (paraventricular, ventromedial nuclei and arcuate nucleus), cortical layer IV, piriform cortex, hippocampus, habenula, dorsal raphe and locus coeruleus. Moderate expression was also detected in the cerebral cortex, thalamus, septum, amygdala, periaquaductal grey and the spinal cord (Mollereau *et al.*, 1994; Bunzow *et al.*, 1994; Wick *et al.*, 1994; Fukuda *et al.*, 1994; Lachowicz *et al.*, 1995; Darland *et al.*, 1998). Extensive immunohistochemical studies conducted by Anton et al. revealed a similar distribution of NOR immunoreactivity in the rat CNS (Anton *et al.*, 1996). Interestingly, light immunoreactivity was detected in the

caudate-putamen, an area in which NOR mRNA had not been detected (Bunzow *et al.*, 1994; Anton *et al.*, 1996). Northern blot analysis indicated that NOR is expressed peripherally in the adrenal gland and liver but is absent in the heart, lung, spleen, intestine and retina (Lachowicz *et al.*, 1995). In contrast, Wang et al. reported NOR expression in the intestine, spleen, vas deferens and liver by reverse transcriptase-PCR (RT-PCR) analysis (Wang *et al.*, 1994). NOR expression has also been detected in murine immune cells, as well as human lymphocytes and lymphocytic cell lines (Wick *et al.*, 1994; Halford *et al.*, 1995; Peluso *et al.*, 1998). Following the isolation of the NOR ligand, Sim et al. used N/OFQ [^{35}S] GTPγS binding studies to localize NOR in rat brain slices (Sim *et al.*, 1997). The results of this study suggest that the cortex, hippocampus, thalamus, amygdala and hypothalamus contained the highest levels of NOR and that NOR has a distinct expression pattern compared with that of the classical opioid receptors (Sim *et al.*, 1997).

Considering the sequence homology of NOR with MOR, DOR and KOR, it was suspected that NOR would be activated by an opioid-like compound. However, several known opioid ligands failed to bind specifically or with high affinity to NOR (Mollereau *et al.*, 1994; Bunzow *et al.*, 1994; Lachowicz *et al.*, 1995). Thus began the race to identify the endogenous ligand for NOR. Using similar strategies, two groups simultaneously identified the ligand (Reinscheid *et al.*, 1995; Meunier *et al.*, 1995). Taking into account its similarity to opioid receptors, NOR was expected to couple to the same second messenger systems. Porcine hypothalamic (Reinscheid *et al.*, 1995) and rat brain (Meunier *et al.*, 1995) extracts were fractionated and screened for their ability to inhibit forskolin-stimulated cAMP production in Chinese Hamster Ovary (CHO) cells heterologously expressing NOR. Both groups reported isolation and purification of the same 17-amino acid protein as the endogenous ligand for NOR (fig.1). Reinscheid et al. named the ligand orphanin FQ since it is the endogenous ligand for an orphan receptor with the amino acids phenylalanine (F) and glutamine (Q) at the N- and C- termini, respectively (Reinscheid *et al.*, 1995). Whereas Meunier et al. termed it nociceptin for its apparent ability to produce hyperalgesia

in mice in the hot plate test (Meunier *et al.*, 1995). For the purpose of clarity, the ligand will be referred to as N/OFQ (nociceptin/orphanin FQ).

The amino acid sequence of N/OFQ is strikingly similar to other opioid peptides, providing evidence for its evolutionary relationship to members of the opioid peptide family (Fig 1). N/OFQ is most homologous to Dynorphin A, which may not be surprising since NOR is most similar to KOR especially in the putative second extracellular loop. However, the dissimilarities among N/OFQ and the other opioid peptides may prove more interesting than the apparent similarities. For example, N/OFQ lacks the canonical N-terminal sequence Y-G-G-F, common to all opioid peptides. The N-terminal N/OFQ sequence (F-G-G-F) differs from that of opioid peptides by a single hydroxyl group.

In addition to similarities in amino acid sequence, structure of the N/OFQ gene is homologous to that of the opioid peptide precursor genes (Mollereau *et al.*, 1996a; Nothacker *et al.*, 1996). The intron-exon boundary within the coding region and the presence of an intron in the 5'-UTR is conserved among the N/OFQ precursor genes and the classical opioid peptide precursor genes (Mollereau *et al.*, 1996a). Moreover, the promotor regions of the N/OFQ precursor gene and the opioid precursor genes contain similar transcriptional regulatory elements (Xie *et al.*, 1999). These data suggest that the N/OFQ precursor gene and the opioid peptide precursor genes have diverged from a common evolutionary ancestor.

N/OFQ is contained within a precursor protein, prepronociceptin/orphanin FQ (ppN/OFQ), with an arrangement similar to that of preprodynorphin, preproopiomelanocortin and preproenkephalin. Dibasic amino acids flank the N/OFQ, suggesting that ppN/OFQ is proteolytically processed to yield bioactive peptides (Mollereau *et al.*, 1996a; Nothacker *et al.*, 1996). Interestingly, the N/OFQ precursor contains other pairs of basic amino acids that may flank additional bioactive peptides. In particular, there is a 28 amino acid peptide immediately downstream of N/OFQ that is 100% conserved among human and murine species. Neither this 28 amino acid peptide nor its

putative cleavage fragments bind NOR, and its biological activity remains to be elucidated (Mollereau *et al.*, 1996a; Nothacker *et al.*, 1996).

Northern blot analysis has revealed that ppN/OFQ is abundantly expressed in the brain and spinal cord. In the rat brain, ppN/OFQ mRNA was particularly abundant in the hypothalamus and striatum and moderately expressed in the hippocampus and cortex (Mollereau *et al.*, 1996a). In situ hybridization studies revealed that the main areas of ppN/OFQ expression in the human brain were in the amygdala and subthalamic nuclei; however, mRNA was also detected in the hypothalamus, substantia nigra, thalamus, corpus callosum and hippocampus (Nothacker *et al.*, 1996). N/OFQ precursor message was essentially undetectable in peripheral tissues, except for low levels of expression in human spleen and rat ovary (Mollereau *et al.*, 1996a; Nothacker *et al.*, 1996). In rat brain, the highest levels of ppN/OFQ mRNA were detected in the bed nucleus of the stria terminalis, lateral septum, central and medial amygdaloid nuclei, dorsal tegmental area and the superior olive by *in situ* hybridization studies (Darland *et al.*, 1998; Nothacker *et al.*, 1996). Moderate ppN/OFQ expression levels were also detected in the arcuate nucleus, preoptic area, zona incerta and the periaquaductal gray (Darland *et al.*, 1998). *In situ* hybridization studies in the mouse CNS demonstrated a similar pattern of ppN/OFQ expression. The highest levels of expression were detected in the central gray, central tegmental field, dorsal division of the nucleus of the lateral lemniscus, superior olive, spinal trigeminal nucleus and the dorsal horn of the spinal cord (Houtani *et al.*, 1996).

N/OFQ peptide distribution has been mapped in the mouse and rat CNS using specific antisera. Immunoreactivity was observed in the dorsal horn of the spinal cord, sensory trigeminal complex, raphe nuclei, locus coeruleus, periaquaductal gray, amygdala, habenula, hypothalamic region and septal area (Schulz *et al.*, 1996). This distribution pattern overlapped that of the opioid peptides in pain modulatory regions, including the superficial dorsal horn, sensory trigeminal complex and the periaquaductal gray. However, opioid and N/OFQ immunoreactivity did not co-localize in single neurons in these regions

(Schulz *et al.*, 1996). Riedl et al. (1996) reported abundant N/OFQ immunoreactivity in the rat spinal cord, including the superficial dorsal horn, lateral spinal nucleus and dorsal gray commissure. These regions are also rich in enkephalin and dynorphin immunoreactivity; however, N/OFQ immunoreactivity did not appear to co-localize with that of enkephalin or dynorphin in nerve terminals or fibers (Riedl *et al.*, 1996). These results suggest that N/OFQ and opioid peptides are released from different nerve terminals and therefore modulate pain signals in a different manner (Schulz *et al.*, 1996; Riedl *et al.*, 1996). Radioimmunoassay has revealed high levels of N/OFQ immunoreactivity in the anterior olfactory nucleus, piriform cortex, central and cortical amygdaloid nuclei, periaquaductal gray and the dorsal spinal cord of the rat (Darland *et al.*, 1998; Schuligoi *et al.*, 1997).

III. Nociceptin/Orphanin FQ Effector Coupling

Given its homology to the opiate receptors, Reinscheid et al. and Meunier et al. correctly predicted that NOR would couple to some of the same second messenger pathways as do the members of the classical opioid receptor family. Indeed, activated NOR leads to inhibition of forskolin-stimulated adenylyl cyclase through a pertussis toxin-sensitive $G_{i/o}$-coupled pathway. In addition to the modulation of adenylyl cyclase activity, NOR has been implicated in the activation of inwardly rectifying K^+ channels and the inhibition of voltage-gated Ca^{2+} channels. Furthermore, NOR can couple to the activation of mitogen activated protein (MAP) kinase and protein kinase C (PKC).

Electrophysiological studies have primarily been carried out on neurons from brain regions known to express high levels of NOR. N/OFQ has been shown to mediate activation of inwardly rectifying K^+ conductance in dorsal raphe (EC_{50}=12 nM) (Vaughan *et al.*, 1996), locus coeruleus (EC_{50}=90 nM) (Connor *et al.*, 1996a), periaquaductal gray (EC_{50}=39 nM) (Vaughan *et al.*, 1997), amygdala (EC_{50}=30.6 nM) (Meis *et al.*, 1998) and

arcuate nucleus (EC_{50}=24 nM) (Wagner *et al.*, 1998) neurons. Naloxone fails to inhibit the N/OFQ mediated K^+ current, with the exception of a weak effect in locus coeruleus neurons. This suggests that the effect is in fact mediated through NOR and not via weak N/OFQ interaction with opioid receptors (Vaughan *et al.*, 1996, 1997; Connor *et al.*, 1996a; Meis *et al.*, 1998). Pertussis toxin prevented N/OFQ-induced K^+ conductance in amygdala neurons, indicating that this response is mediated via a $G_{i/o}$ protein (Meis *et al.*, 1998).

N/OFQ has been shown to modulate a number of different types of voltage-gated Ca^{2+} currents. Connor et al. demonstrated that N/OFQ inhibits N-type Ca^{2+} channels in the human neuroblastoma SH-SY5Y cells with an IC_{50} value of 42 nM (Connor *et al.*, 1996b). The effects are sensitive to pertussis toxin and insensitive to the opiate antagonists naloxone, CTAP and naltrindole, providing evidence for the involvement of a $G_{i/o}$ protein and that this effect is not opioid receptor mediated (Connor *et al.*, 1996b). In freshly dissociated CA1 and CA3 hippocampal neurons, N/OFQ modulated activity of the three major types of Ca^{2+} channels, L-, N- and P/Q (Knoflach *et al.*, 1996). These effects could not be accounted for by opioid receptors because naloxone failed to reverse the N/OFQ effect. As in the SH-SY5Y cells, a $G_{i/o}$ protein appears to be involved as the effect was blocked by pertussis toxin pretreatment and was irreversible in the presence of GTPγS (Knoflach *et al.*, 1996). Coupling of NOR to inwardly rectifying K^+ channels and N-type Ca^{2+} channels are two features it shares with the classical opioid receptors. However, unlike opiates, N/OFQ inhibits a T-type Ca^{2+} channel current in dorsal root ganglion neurons by a G protein-independent mechanism (Abdulla *et al.*, 1997). GTPγS, GDPβS or aluminum fluoride failed to modulate this low-voltage-activated transient Ca^{2+} current, suggesting a lack of G protein involvement (Abdulla *et al.*, 1997).

N/OFQ has been shown to activate protein kinase C (PKC) (Lou *et al.*, 1997) and the mitogen-activated protein kinase (MAPK) pathway (Lou *et al.*, 1998; Hawes *et al.*, 1998) which is involved in the regulation of cell growth and proliferation. The mode of MAPK activation via G-proteins may be mediated through a pathway that utilizes the βγ

subunit of G_i and a multi-protein complex that ultimately activates $p21^{ras}$. Alternatively, the G_o protein may stimulate MAPK activity through a PKC-dependent pathway. In CHO cells stably expressing NOR, Lou et al. (1998) demonstrated that N/OFQ mediates the activation of the MAPK pathway in a concentration-dependent manner with maximal activation about 5.5 times that of basal activity levels. This effect was blocked by pertussis toxin and was naloxone insensitive. Furthermore, specific inhibitors of PKC and phospholipase C (PLC) suppressed N/OFQ-stimulated MAPK activity, suggesting that PKC and PLC are critically involved in this N/OFQ-mediated signaling cascade (Lou *et al.*, 1998). Although PLC activity may be mediated via opioid receptor activation (Smart *et al.*, 1995, 1996), it has been suggested that the classical opioid receptors stimulate MAPK activity in a PLC-independent manner (Fukuda *et al.*, 1996). This difference in signaling cascades may be critical in understanding the different effects mediated by the N/OFQ system compared with that of the classical opioid receptor systems. A similar study conducted by Hawes et al. confirmed pertussis toxin sensitive, concentration-dependent N/OFQ-mediated MAPK activation with maximal stimulation about 3.2-times basal levels (Hawes *et al.*, 1998). This activity was attenuated by specific inhibitors targeted at $G\beta\gamma$ subunits, son of sevenless (sos), and phosphotidylinositol 3-kinase (PI-3K), putative components of the G_i-coupled pathway (Hawes *et al.*, 1998). Additionally, inhibition or cellular depletion of PKC had no effect on this N/OFQ-mediated stimulation of MAPK, suggesting that like the classical opioid receptor-mediated MAPK stimulation, this pathway was PKC-independent and G_i-protein mediated (Hawes *et al.*, 1998). The results of these two studies may appear contradictory; however, in the report by Lou et al., treatment with a PKC-specific inhibitor did not completely block N/OFQ-induced MAPK activation (Lou *et al.*, 1998). This suggests that NOR may stimulate MAPK activity through both G_i- and G_o-proteins in this cell culture system.

IV. Nociceptin/Orphanin FQ Pharmacology

Despite the predicted structural and sequence homology between NOR and the classical opioid receptors and between N/OFQ and opioid peptides, they do not overlap pharmacologically. Ligands that bind to opioid receptors with high affinity fail to bind to NOR with high affinity (Mollereau *et al.*, 1994; Bunzow *et al.*, 1994). Moreover, N/OFQ does not bind to the opioid receptors with appreciable affinity (Reinscheid *et al.*, 1995). Further pharmacological evidence that the NOR-N/OFQ system is distinct from the opioid system includes the negligible affinity of the non-selective opioid agonists etorphine and bremazocine and the antagonists naloxone and nalorphine for NOR (Mollereau *et al.*, 1994).

With the pharmacological characterization of NOR, several groups have reported binding affinities of N/OFQ for NOR that vary considerably. Indeed, in rat brain membranes, reported K_d values range from about 20 pM (Albrecht *et al.*, 1998) to greater than 5 nM (Dooley *et al.*, 1996), suggesting the need for a standardized receptor binding assay. Stable GTP analogues or Na^+ reduced N/OFQ binding affinity for NOR in tissues, as well as in cell culture, consistent with the notion that NOR is G protein-coupled (Albrecht *et al.*, 1998; Ardati *et al.*, 1997; Makman *et al.*, 1997). In cell lines stably expressing NOR, Ardati et al. reported virtually identical binding constants for $[^3H]$N/OFQ labeled at L^{14} and the Y^{14}-substituted $[^{125}I\text{-}Y^{14}]$ N/OFQ (Ardati *et al.*, 1997). In rat cerebral cortex, $[^3H\text{-}Y^{14}]$-N/OFQ and $[I^{125}\text{-}Y^{14}]$-N/OFQ exhibited a five-fold difference in binding affinity, 22 pM and 117 pM, respectively (Albrecht *et al.*, 1998). Cloned mouse, rat and human NOR expressed in CHO cells exhibit similar binding constants in spite of the position or type of the radioactive label (Reinscheid *et al.*, 1995, 1996; Dooley *et al.*, 1997; Pan *et al.*, 1996; Butour *et al.*, 1997). The neuroblastoma cell lines, SK-N-SH and NG108-15, endogenously express NOR and appear to have nanomolar binding affinities for N/OFQ (Cheng *et al.*, 1997; Ma *et al.*, 1997). Table 1 summarizes some of the pharmacological

studies of NOR in tissues and cell lines with differentially labeled ligands. The N/OFQ binding assay has been confounded by N/OFQ peptide instability and, until recently, by the lack of a high-affinity small molecule agonist or reliable antagonist.

Because N/OFQ is sensitive to degradation a small molecule agonist that acts at NOR will be expected to be more stable and yield more reliable results in pharmacological and behavioral studies. Furthermore, a non-peptide agonist will be suitable for intraperitoneal or oral administration whereas N/OFQ must be administered i.c.v. to observe *in vivo* effects. Wnendt et al. developed a reporter gene assay for high throughput screening of compounds for the identification of NOR agonists and antagonists (Wnendt, *et* al., 1999). Using this reporter gene assay, buprenorphine was shown to act as a full agonist at NOR with an IC_{50} of 8.4 nM, about 10-times that of N/OFQ (Wnendt *et al.*, 1999). In a recent report, two non-peptidyl, high-affinity, potent NOR receptor agonists have been identified (Röver *et al.*, 2000). The compounds, designated 1p and 1q, are substituted 1-phenyl-1,3,8-triazaspiro[4.5]decan-4-ones and bear some resemblance to the opioid compound lofentanil (Röver *et al.*, 2000). The compounds exhibit selectivity for NOR compared with opioid receptors and act as full agonists in GTPγS binding assays (Röver *et al.*, 2000).

Several groups have attempted to identify a selective and potent antagonist at NOR. A modified N/OFQ peptide, $[Phe_1\Psi(CH_2\text{-}NH)Gly_2]N/OFQ(1\text{-}13)NH_2$, displaced the concentration-response curves of N/OFQ in electrically stimulated guinea pig ileum and rat vas deferens (Guerrini *et al.*, 1998; Calò *et al.*, 1998). Moreover, the peptide showed no effect on these tissues when applied alone and did not displace the concentration-response curves mediated through MOR, DOR or KOR (Guerrini *et al.*, 1998; Calò *et al.*, 1998). These results suggested that $[Phe_1\Psi(CH_2\text{-}NH)Gly_2]N/OFQ(1\text{-}13)NH_2$ is a competitive and selective antagonist at NOR and is inactive at opioid receptors (Guerrini *et al.*, 1998; Calò *et al.*, 1998). However, in CHO cells expressing human NOR, this peptide was found to potently inhibit forskolin-stimulated adenylyl cyclase activity (IC_{50} = 7.5 nM) (Butour *et al.*,

1998). This result suggests that $[Phe_1\Psi(CH_2\text{-}NH)Gly_2]N/OFQ(1\text{-}13)NH_2$ can act as an agonist at NOR in this cell line. The authors suggest that the different action of the peptide in smooth muscle preparations compared with CHO cells may be due to activation of different receptor types (Butour *et al.*, 1998). Grisel et al. demonstrated that *in vivo* $[Phe_1\Psi(CH_2\text{-}NH)Gly_2]N/OFQ(1\text{-}13)NH_2$ dose-dependently inhibited morphine analgesia in mice, an effect also observed with N/OFQ administration (Grisel *et al.*, 1998). Furthermore, the peptide fails to antagonize the anti-opioid action of N/OFQ (Grisel *et al.*, 1998). In conscious rats, intracerebralventricular (i.c.v.) administration of $[Phe_1\Psi(CH_2\text{-}NH)Gly_2]N/OFQ(1\text{-}13)NH_2$ dose-dependently induces similar cardiovascular and renal effects as does N/OFQ (Kapusta *et al.*, 1999). Moreover, i.c.v. pretreatment with a low (inactive) dose of $[Phe_1\Psi(CH_2\text{-}NH)Gly_2]N/OFQ(1\text{-}13)NH_2$ failed to block cardiovascular and renal responses evoked by i.c.v. administration of N/OFQ (Kapusta *et al.*, 1999). Taken together, these data indicate that $[Phe_1\Psi(CH_2\text{-}NH)Gly_2]N/OFQ(1\text{-}13)NH_2$ has agonistic, not antagonistic, activity at NOR *in vitro* as well as *in vivo*.

The hexapeptide acetyl-Arg-Tyr-Tyr-Arg-Ile-Lys-NH_2 (Ac-RYYRIK-NH_2) has been shown to act as a partial agonist at NOR in GTPγS binding assays *in vitro* (Dooley *et al.*, 1997). In contrast, *in vivo* the peptide antagonizes N/OFQ-stimulated $[S^{35}]$ GTPγS binding to G proteins in rat brain membranes and sections (Berger *et al.*, 1999). This antagonism at NOR was competitive, high affinity, and specific to NOR as Ac-RYYRIK-NH_2 failed to inhibit agonist stimulated GTPγS binding at MOR, DOR and KOR (Berger *et al.*, 1999). Moreover, Ac-RYYRIK-NH_2 suppresses the N/OFQ-mediated chronotropic effect on neonatal rat cardiomyocytes (Berger *et al.*, 1999). It is interesting that these compounds behave differently, agonistic or antagonistic, depending on the system, further delineating the distinct pharmacology and physiology of *in vitro* and *in vivo* systems.

Table 1

Tissue/Cell Type	Ligand	K_d	Reference
CHO: rat NOR	^{125}I-Y^{14} N/OFQ	0.1 ± 0.02 nM	Reinscheid *et al.* 1995
CHO: rat NOR	^{125}I-Y^{14} N/OFQ	56.2 ± 7.3 pM	Reinscheid *et al.* 1996
CHO: mouse NOR	^{125}I-Y^{14} N/OFQ	36.7±1.6 pM	Pan *et al.* 1996
CHO: mouse NOR	^{3}H-P^{1}, P^{4} N/OFQ	0.15 nM	Dooley *et al.* 1997
CHO: human NOR	^{3}H-L^{14} N/OFQ	0.11 ± 0.03 nM	Butour *et al.* 1997
HEK: rat NOR	^{125}I-Y^{14} N/OFQ ^{3}H-L^{14} N/OFQ	469 ± 64 pM 229 ± 56 pM	Ardati *et al.* 1997
SK-N-SH (human neuroblastoma)	^{3}H-P^{1}, P^{4} N/OFQ	1.3 ± 0.1 nM	Cheng *et al.* 1997
NG108-15 hybrid (neuroblastoma x glioma)	^{3}H-P^{1}, P^{4} N/OFQ	3.6 ± 0.6 nM	Ma *et al.* 1997
rat brain	^{3}H-P^{1}, P^{4} N/OFQ	5 ± 1.1 nM	Dooley and Houghten 1996
rat cerebral cortex	^{3}H-Y^{14} N/OFQ ^{125}I-Y^{14} N/OFQ	21.6 ± 8.2 pM 116.7 ± 5.08 pM	Albrecht *et al.* 1998
rat hypothalamus	^{125}I-Y^{14}	103 ± 20 pM	Makman *et al.* 1997
human fetal hypothalamus	^{125}I-Y^{14} N/OFQ	215 ± 59 pM	

Y = tyrosine, P = phenylalanine, L = leucine

Recently, the small molecule 1-[(3R,4R)-1-Cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2H-benzimidazol-2-one, termed J-113397, has been identified as a potent and selective antagonist for NOR (Kawamoto *et al.*, 1999; Ozaki *et al.*, 2000). J-113397 was shown to inhibit [^{125}I] [Y^{14}] N/OFQ binding to NOR expressed in CHO cells with an IC_{50} of 2.3 nM and to possess low affinity for MOR, DOR and KOR (IC_{50} > 1000 nM) (Kawamoto *et al.*, 1999). In GTPγS studies this acts as a full antagonist (IC_{50} 5.6 nM) and not as an agonist (EC_{50} >10,000 nM) (Kawamoto *et al.*, 1999). The J-113397 compound reverses the N/OFQ mediated inhibition of forskolin-stimulated cAMP accumulation in CHO cells expressing hNOR (Ozaki *et al.*, 2000). This J-113397 effect was dose-dependent, yielding an IC_{50} value of 26 ± 3.1 nM (Ozaki *et al.*, 2000). These results suggest that J-113307 is a potent and selective non-peptidyl antagonist at NOR (Kawamoto *et al.*, 1999; Ozaki *et al.*, 2000), however, subsequent studies are necessary to confirm antagonistic activity *in vivo*.

IV. Mutagenesis of NOR and Nociceptin/Orphanin FQ

Site-directed mutants and chimeric forms of NOR have been constructed in an effort to determine critical amino acids and receptor domains involved in N/OFQ binding. Considering that NOR is most closely related to KOR, Meng et al. (1996) mutated four specific NOR amino acids which resulted in a receptor that bound both N/OFQ and pro-dynorphin products with subnanomolar affinity. The four residues, located near the extracellular surface of transmembrane domains (TMD) VI and VII, were mutated to residues that are conserved in MOR, DOR and KOR (Meng *et al.*, 1996). The authors interpret their findings as support for the hypothesis that the amino acid residues of NOR that are important for excluding opioids are not involved in N/OFQ binding (Meng *et al.*, 1996). In a follow-up study, an additional amino acid residue located at the extracellular

face of TMD V/second extracellular loop of NOR was mutated to determine whether this would create an opiate binding site (Meng *et al.*, 1998). Not only does the opiate alkaloid naltrindole exhibit high affinity for this mutated NOR, naloxone, nor-binaltrophine HCL (nor-BNI) and (-)-bremazocine show increased affinity for this mutant receptor compared with the wild-type NOR (Meng *et al.*, 1998). This mutant NOR exhibits etorphine stimulated [^{35}S]GTP binding, however, N/OFQ fails to activate this mutant NOR in [^{35}S]GTP binding studies (Meng *et al.*, 1998). Moreover, the etorphine-stimulated [^{35}S]GTP binding mediated via this mutant NOR was blocked by naltrindole, suggesting that the agonistic and antagonistic functions of these compounds are retained at this receptor (Meng *et al.*, 1998).

Chimeric NORs containing the N-terminus and TMD 1 of either MOR or DOR exhibited greatly increased affinity for naloxone benzoylhydrazone (NalBzoH) compared with that of the wild type NOR (Pan *et al.*, 1996). N/OFQ exhibited equal binding affinity for the MOR/NOR chimera and the wild type NOR (Kd = about 38 pM) but showed a slight reduction in affinity for the DOR/NOR chimera (Kd = 135 pM) (Pan *et al.*, 1996). These studies suggest that the N-terminus and first TMD of NOR are not critical for high affinity N/OFQ binding, but exclude binding of naloxone benzoylhydrazone (NalBzoH) (Pan *et al.*, 1996).

In an effort to identify amino acid residues critical for high affinity binding to NOR, alanine scanning mutagenesis of N/OFQ was performed (Reinscheid *et al.*, 1996; Dooley *et al.*, 1996). Each N/OFQ amino acid residue was individually substituted with alanine (A) and the binding affinity of the resultant peptide for NOR was determined. This analysis revealed that F1, F4 and R8 are the most crucial amino acid residues with respect to high affinity binding to NOR, and A substitution of G2 also results in a notable reduction in binding affinity (Dooley *et al.*, 1996; Reinscheid *et al.*, 1996). In contrast, A substitution of R10, L14 and Q16 had no significant effect on binding affinity (Dooley *et al.*, 1996; Reinscheid *et al.*, 1996). Additionally, substituting N/OFQ residues 1-8 with D-amino acids

results in reduced affinity for NOR, with the exception that D-A2 N/OFQ and D-A7 N/OFQ retain high affinity for NOR (Reinscheid *et al.*, 1996). Functional studies were also carried out with these substituted peptides. N/OFQ residues 1,2,4,5 and 8 appeared to be most critical for receptor activation in the A substituted peptides (Reinscheid *et al.*, 1996). N/OFQ D-amino acid substitutions that resulted in the greatest reduction in functional efficacy at NOR were [D-F1], [D-A3], [D-F4], [D-T5], [D-A7] and [D-R8] (Reinscheid *et al.*, 1996). These results suggest that the N-terminus of N/OFQ plays a more critical role in binding and function than does the C-terminus (Reinscheid *et al.*, 1996; Dooley *et al.*, 1996).

Truncation mutagenesis of the amino- and carboxy- termini of N/OFQ was also performed in an effort to determine amino acids that are critical for receptor binding and activation. The shortest N/OFQ N-terminal fragment that retained high-affinity for NOR was N/OFQ 1-13. Further C-terminal truncation of N/OFQ resulted in a marked reduction in binding affinity for NOR (Butour *et al.*, 1997; Dooley *et al.*, 1996). The N/OFQ 1-13 fragment, however, exhibited only 1/30th the potency of N/OFQ in inhibiting forskolin-stimulated cAMP accumulation (Butour *et al.*, 1997). Since the Y residue at position one is conserved in all opioid peptides, the F1 of N/OFQ was substituted with Y to examine the importance of this residue for binding and activation of NOR (Reinscheid *et al.*, 1996; Butour *et al.*, 1997; Shimohigashi *et al.*, 1996). [Y1]N/OFQ retained binding affinity for NOR and functional potency in cAMP studies (Reinscheid *et al.*, 1996; Butour *et al.*, 1997). Moreover, [Y1]-N/OFQ retained behavioral effects in tail-flick assays (Shimohigashi *et al.*, 1996). Carboxy terminal N/OFQ fragments, N/OFQ 2-17, N/OFQ 6-17 and N/OFQ 12-17, showed reduced binding affinity for NOR (Butour *et al.*, 1997). Surprisingly, N/OFQ fragments 6-17 and 12-17 retained about 10-fold higher binding affinity than did N/OFQ 2-17 (Butour *et al.*, 1997). However, none of these amino terminally truncated fragments retained functional potency (Butour *et al.*, 1997).

The amino acid sequence of N/OFQ contains two putative dibasic amino acid (RK) cleavages sites at positions 8,9 and 12,13 (fig.1). N/OFQ fragments that could result from cleavage at these putative sites may mediate biological activity at NOR or another, yet unidentified receptor. Several groups have investigated binding affinity and functional activity of putative N/OFQ cleavage products at NOR (Reinscheid *et al.*, 1996; Shimohigashi *et al.*, 1996; Mathis *et al.*, 1997, 1998; Rossi *et al.*, 1997; Calò *et al.*, 1996; Guerrini *et al.*, 1997). The fragments N/OFQ 1-11 and N/OFQ 1-7 failed to bind NOR with appreciable affinity (Reinscheid *et al.*, 1996; Shimohigashi *et al.*, 1996) or activate NOR (Reinscheid *et al.*, 1996) in cell lines stably expressing the receptor. These results suggest that, in contrast to dynorphin A, removal of C-terminal amino acids renders N/OFQ inactive (Reinscheid *et al.*, 1996; Shimohigashi *et al.*, 1996). In mouse brain homogenates, both N/OFQ 1-7 and N/OFQ 1-11 exhibit low affinity binding (Mathis *et al.*, 1997). Strikingly, N/OFQ 1-11 inhibits forskolin-stimulated cAMP production in mouse brain homogenates in a dose-dependent manner with an IC_{50} value similar to that of N/OFQ (Mathis *et al.*, 1997). In contrast, N/OFQ 1-7 failed to show any functional activity in these studies (Mathis *et al.*, 1997). Moreover, N/OFQ 1-11 produced analgesia when injected i.c.v. in mice (Meng *et al.*, 1998; Rossi *et al.*, 1997). The observed differences in putative N/OFQ 1-11 activity in tissues compared with cell lines suggests that N/OFQ 1-11 may function through an unidentified receptor and not at NOR. Other putative N/OFQ fragments, N/OFQ 1-13 as well as N/OFQ 1-13-NH_2, have been shown to reduce the number of twitches in electrically-stimulated mouse vas deferens in a concentration-dependent manner (Calò *et al.*, 1996; Guerrini *et al.*, 1997). N/OFQ 1-13-NH_2 was found to be as active as N/OFQ in this bioassay, suggesting that the entire N/OFQ sequence is not required for biological activity in some tissue preparations (Guerrini *et al.*, 1997). Furthermore, N/OFQ 1-13-NH_2 retains higher activity at NOR compared with non-amidated N/OFQ 1-13, presumably due to its protection from carboxypeptidases (Guerrini *et al.*, 1997). The N-terminal amino acids F-G-G-F and the basic residues (R8,12 ; K9,13)

appear to be essential for N/OFQ's ability to activate NOR because replacing any one of them with A results in an inactive peptide (Guerrini *et al.*, 1997). Moreover, F4 and not F1 appears to be the critical residue for receptor activation (Guerrini *et al.*, 1997). Collectively these results indicate that the shortest N/OFQ fragment that retains considerable binding affinity and functional activity at NOR is N/OFQ1-13 and that this peptide's stability and efficacy are enhanced when amidated at its C-terminus.

VI. Nociceptin/Orphanin FQ Metabolism and Degradation

N/OFQ metabolism and the possible functional significance of the resulting peptides have been investigated by several groups. In human blood, the predominant site where N/OFQ is cleaved is the peptide linkage F1-G2, resulting in N/OFQ 2-17 (Yu *et al.*, 1996). This putative biotransformation pathway is similar to that of dynorphin A 1-17. However, N/OFQ appears to be more resistant to biotransformation than does dynorphin A 1-17 (Yu *et al.*, 1996). In SHSY-5Y neuroblastoma and a human lung carcinoma cell line, as well as in primary cortical cells, the major N/OFQ metabolic products were the amino-terminal peptides, N/OFQ 1-9 and N/OFQ 1-13, suggesting that cleavage may occur at paired basic amino acids (RK) (Vlaskovska *et al.*, 1999). This appears to be mediated via a metallosensitive endopeptidase since cleavage was effectively inhibited by EDTA, PMSF, Hg^{2+}, Cu^{2+} and Zn^{2+} (Vlaskovska *et al.*, 1999). Additionally, cleavage was inhibited in the presence of dynorphin A 2-17, suggesting that the same proteolytic enzyme recognizes both N/OFQ and dynorphin A 2-17 (Vlaskovska *et al.*, 1999). Pre-treatment of these cultured cells with morphine results in increased accumulation of the N/OFQ metabolites, N/OFQ 1-9 and N/OFQ 1-13 as well as accumulation of N/OFQ 1-12, N/OFQ 1-6 and N/OFQ 1-5 (Vlaskovska *et al.*, 1999). This effect of morphine is mediated via an opioid receptor since it is naloxone sensitive (Vlaskovska *et al.*, 1999).

N/OFQ processing has also been studied in spinal cord and brain tissues (Suder *et al.*, 1999; Montiel *et al.*, 1997). Suder et al. demonstrated in rat spinal cord that the biotransformation of N/OFQ is primarily mediated by a neutral serine endopeptidase initially resulting in the product N/OFQ 1-11. This peptide is further reduced to what appears to be the final processed product N/OFQ 1-6 (Suder *et al.*, 1999). In the hot plate test N/OFQ 1-6 produces a bi-phasic effect in rats: the production of analgesia 10 min following i.c.v. injection that is succeeded by hyperalgesia 30 min post-injection (Suder *et al.*, 1999). These behavioral effects are effectively inhibited by naloxone and NMDA, respectively (Suder *et al.*, 1999). The results of this study suggest that N/OFQ fragments may retain biological activity, which may be mediated through a receptor other than NOR (Suder *et al.*, 1999). In the presence of mouse cortical tissue, N/OFQ is predominately cleaved at peptide bonds F1-G2, A7-R8, A11-R12 and R12-K13 by aminopeptidase N and endopeptidase 24.15 (Montiel *et al.*, 1997). Endopeptidase 24.11 (enkephalinase), however, does not appear to play a role in this metabolism (Montiel *et al.*, 1997). Interestingly, EDTA abolishes this cleavage, indicating metallo-sensitive protease activity (Montiel *et al.*, 1997). *In vivo*, Noble and Roques (1997) investigated the effects of i.c.v. co-administration of N/OFQ and endopeptidase 24.15 and aminopeptidase N inhibitors. Presence of the inhibitors potentiates the N/OFQ mediated effects on motor activity, providing evidence for *in vivo* involvement of these peptidases in N/OFQ inactivation (Noble and Roques, 1997). Metabolism of N/OFQ was examined *in vivo* in the rat hippocampus (Sandin *et al.*, 1999). Metabolism proceeds in a step-wise fashion, first yielding the products N/OFQ 1-13 and N/OFQ 14-17. The former is further metabolized to N/OFQ 1-9 and N/OFQ 10-13 (Sandin *et al.*, 1999). That the products N/OFQ 1-9 and N/OFQ 1-13 have identical C-termini suggests that the cleavages releasing these two fragments may be mediated by the same enzyme (Sandin *et al.*, 1999). In contrast with N/OFQ, N/OFQ 1-13 fails to affect motor function and spatial learning when injected into rat hippocampus (Sandin *et al.*, 1999).

In addition to examination of N/OFQ *in vivo* and *in vitro* the integrity of the [^{3}H] labeled N/OFQ ligand has been examined (Quigley *et al.*, 2000). It has been our experience that [^{3}H] N/OFQ peptides labeled at positions F1 and F4 have decreased inherent stability compared with peptides labeled at position L14. Moreover, the integrity of the peptide was severely compromised in the presence of cell membranes, suggesting that enzymatic activity can contribute to the instability of the peptide during binding assays. That the peptide remains intact in the presence of cellular membranes pre-treated at low pH provided further support for the idea that enzymatic activity mediates cleavage of [^{3}H] N/OFQ. Consequently, it was surprising that we were unable to block [^{3}H] N/OFQ degradation with protease inhibitors including amino peptidase and endopeptidase inhibitors and non-specific protease inhibitors or by a proteosome inhibitor (Quigley *et al.*, 2000). Therefore, to establish a reliable and reproducible binding protocol, consideration of ligand integrity is imperative and may in fact account for some of the variability in binding results reported in the literature.

VII. Summary

Based on a number of criteria including sequence similarity, gene structure, second messenger coupling and similarity of peptide ligand sequence, NOR appears to belong to the family of opioid receptors. Indeed, the sequence similarity with opioid receptors was the basis for its cloning. However, NOR is not an opioid receptor. It does not bind opioid ligands with high affinity and pharmacological effects mediated by NOR-N/OFQ binding are insensitive to the prototypical opiate antagonist naloxone. N/OFQ shares extensive sequence homology with opioid peptides, particularly dynorphin A. However, as is the case with NOR and opioid receptors, the similarity between N/OFQ and the opioid peptides appears to be limited to sequence.

The involvement of N/OFQ in mediating numerous behavioral effects has been implied. However, in contrast with opioid peptides, N/OFQ does not appear to produce analgesia supraspinally. Explaining the reason for the evolutionary relationship between the N/OFQ system and the opioid system has been a difficult task. That the primary role of N/OFQ in the brain is to mediate anti-opioid functions and to maintain homeostasis is an attractive hypothesis, which may account for the conserved sequence similarities between the receptors and endogenous peptides. However, this hypothesis does not necessarily account for the numerous behavioral effects attributed to N/OFQ.

One possible explanation for the diversity of N/OFQ effects may be the putative activity of N/OFQ metabolic products. Proteases that mediate N/OFQ metabolism are active *in vivo* as N/OFQ metabolism has been demonstrated in spinal cord and hippocampus (Suder *et al.*, 1999; Sandin *et al.*, 1999). Furthermore, N/OFQ 1-6, a putative metabolic fragment produced in the spinal cord mediates behavioral effects when injected i.c.v. in rats (Suder *et al.*, 1999). Since most N/OFQ fragments fail to bind or activate NOR with high affinity or appreciable potency, the functionality of an N/OFQ metabolic product suggests the existence of unidentified receptors. Future studies are necessary to determine the importance of putative N/OFQ degradation products and identify the receptors mediating the functional effects of these products. Of course, one should not overlook the obvious possibility that the purpose of N/OFQ metabolism is also to abolish the activity of N/OFQ *in vivo*.

That morphine potentiates the metabolism of N/OFQ in cultured cell lines suggests a functional link between the opioid system and N/OFQ (Vlaskovska *et al.*, 1999). This observation supports the hypothesis that N/OFQ may function primarily as an anti-opioid peptide. One might expect that the opioid and N/OFQ systems functionally antagonize one another, therefore activation of the opioid system by administration of morphine might be expected to inhibit the activity of N/OFQ, perhaps by stimulating N/OFQ metabolism. This

idea also suggests that the purpose of N/OFQ metabolism is to block N/OFQ activity, not necessarily to induce alternative functional effects of N/OFQ metabolic products.

The fact that NOR and N/OFQ exhibit widespread distribution in the CNS suggests that this neuropeptide-receptor system may be involved in a number of behavioral functions that are not limited to interactions with the opioid system. We developed a radioimmunoassay (RIA) for detection of the peptide in tissues in an effort to begin to elucidate functional roles for N/OFQ *in vivo*. Our survey of central and peripheral tissues indicates an abundance of N/OFQ immunoreactivity in the mouse hypothalamus. This result suggested that *in vivo* N/OFQ may play a functional role in hypothalamic-pituitary-adrenal (HPA) axis activity and the response to stress. To test this hypothesis, we measured changes in mouse hypothalamic N/OFQ immunoreactivity levels in response to modulation of the HPA axis. The results of these studies suggested that chronic restraint stress and administration of the synthetic glucocorticoid, dexamethasone, leads to a reduction in hypothalamic N/OFQ immunoreactivity. Furthermore, transgenic mice that over-produce corticotrophin releasing hormone (CRH) demonstrated elevated levels of hypothalamic N/OFQ immnuoreactivity. Hypothalamic β-endorphin immunoreactivity was unaltered by these physiological effects on HPA axis activity. Moreover, Hypothalamic N/OFQ immunoreactivity levels were unaltered in transgenic mice that lack β-endorphin. Taken together these results suggest that N/OFQ appears to be affected by activity of the HPA axis, which may be independent of N/OFQ interaction with the opioid system.

While examining the effects of HPA axis activity on hypothalamic N/OFQ immunoreactivity levels we identified physiological conditions that resulted in a significant reduction or elevation of hypothalamic N/OFQ. We became interested in how the N/OFQ peptide mediates its activity at NOR and the molecular pharmacology of this peptide-receptor system. Mutant forms of NOR were created via site-directed mutagenesis. Specifically, aspartic acid 107 (D107) in transmembrane domain (TMD) II and arginine 299 (R299) in TMD VII were mutated to alanine. These amino acids were chosen based on

a model for activation of the rhodopsin and α_{1b}-adrenergic receptors. We hypothesized that D107 and R299 my form an interhelical salt-bridge linkage that constrains the receptor in the inactive conformation. Disruption of this salt-bridge linkage is thought to be sufficient for receptor activation. Indeed, mutation of homologous amino acids in the rhodopsin or α_{1b}-adrenergic receptors, such that salt-bridge formation is disrupted, results in constitutive activity of the mutant receptors. Based on this hypothesis, three NOR mutants were created, NOR D107A, NOR R299A and NOR D107A/R299A. These NOR mutants were then examined for N/OFQ binding affinity and functional activity in coupling to second messenger systems.

In the process of evaluating the binding affinity of the mutant NORs for N/OFQ, we determined that the tritiated N/OFQ peptide is very sensitive to degradation. Reversed phase-high performance liquid chromatography (RP-HPLC) analysis of N/OFQ tritiated at amino acid positions Phe^1 and Phe^4 revealed that this peptide is unstable in storage. Reliable and reproducible NOR binding results were not obtained with this labeled peptide. Next, we obtained N/OFQ peptide tritiated at amino acid postion Leu^{14}. RP-HPLC analysis revealed that this peptide is stable in storage for up to several months. In spite of the intrinsic stability of $[^3H]Leu^{14}$-N/OFQ, this peptide was labile when incubated in the presence of cellular material, as in the NOR binding assay. The putative enzymatic degradation of $[^3H]Leu^{14}$-N/OFQ in the presence of cell membranes could not be completely blocked by various combinations of protease inhibitors. However, reproducible NOR binding results were obtained with this peptide, suggesting that the NOR possesses high affinity for $[^3H]Leu^{14}$-N/OFQ such that receptor binding is achieved in spite of enzymatic cleavage of the peptide.

Once we established a reliable NOR-$[^3H]Leu^{14}$-N/OFQ binding protocol, the mutant forms of NOR were evaluated for $[^3H]Leu^{14}$-N/OFQ binding affinity and compared with that of the wild-type NOR. NOR R299A demonstrated increased binding affinity while NOR D107A and NOR D107A/R299A exhibited markedly reduced binding affinity

for $[^3H]Leu^{14}$-N/OFQ compared with that of the wild-type NOR. Functional efficiency of the mutant NORs was determined by cAMP assays. NOR has been shown to couple to inhibition of the adenylyl cyclase enzyme resulting in a dose-dependent N/OFQ-mediated reduction in intracellular cAMP accumulation. Compared with the wild-type NOR, the NOR R299A possessed slightly reduced functional potency. Conversely, the functional potency of the D107A and D107A/R299A NOR mutants was significantly reduced compared with that of the wild-type receptor. The NOR mutants do not appear to be constitutively active which suggests that activation of NOR may not be mediated via disruption of a salt-bridge linkage between residues D107 and R299. However, it is an interesting observation that the NOR R299A mutation resulted in increased affinity for $[^3H]Leu^{14}$-N/OFQ, yet reduced functional potency of the receptor. These seemingly paradoxical results suggest that NOR binding and activation may be mediated via different mechanisms.

The studies presented in this dissertation begin to elucidate the physiology and pharmacology of the NOR-N/OFQ system. They represent the development of a radioimmunoassay (RIA) for N/OFQ, the utility of the N/OFQ RIA in physiological studies, the evaluation of N/OFQ stability and some molecular pharmacological studies of NOR-N/OFQ interactions. This series of experimental studies is a valuable contribution to the NOR-N/OFQ field in that the RIA will be a useful tool for detection of the N/OFQ peptide. The studies of N/OFQ stability serve to caution others regarding proper handling and storage of the peptide. Furthermore, the molecular pharmacological studies of NOR-N/OFQ presented here indicated NOR amino acid residues that may be involved in N/OFQ binding and suggests the importance of NOR-N/OFQ charge interactions for receptor binding and activation. The collection of data represented in this dissertation establishes a foundation for future physiological and pharmacological studies of the NOR-N/OFQ system.

Chapter 1

Orphanin FQ is the Major OFQ^{1-17}-containing Peptide Produced in the Rodent and Monkey Hypothalamus

Orphanin FQ is the Major OFQ$^{1-17}$-containing Peptide Produced in the Rodent and Monkey Hypothalamus

Denise I. Quigley[#], John McDougall, Tristan Darland[†], Ge Zhang, Oline R*nnekleiv[@]
David K. Grandy, and Richard G. Allen*
Vollum Institute for Advanced Biomedical Research, *The Center for Research on Occupational and Environmental Toxicology, #Molecular and Medical Genetics † Cell and Developmental Biology, Oregon Health Sciences University Portland, Oregon 97201, @ The Oregon Regional Primate Research Center

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Corresponding Author:
Richard G. Allen, Ph.D.
CROET L-606
Oregon Health Sciences University
3181 SW Sam Jackson Park Road
Portland, OR 97201
Phone: (503) 494-2508
FAX: (503) 494-6831
E-mail: allenr@ohsu.edu

Running title: Orphanin FQ processing in the hypothalamus

Abstract

In order to investigate the processing of OFQ containing peptides in the hypothalamus we have developed a sensitive and quantitative radioimmunoassay for OFQ. We fractionated rodent and monkey hypothalamic extracts by reversed-phase high performance liquid chromatography and found that the extracts contained multiple peaks of OFQ immunoreactivity with the major peak co-eluting with synthetic OFQ^{1-17}. Mouse hypothalamic extracts were also fractionated by SDS-PAGE to determine the apparent molecular weights of molecules containing the OFQ peptide. Multiple peaks of OFQ immunoreactivity, ranging in size from approximately 1 to 30 kilodaltons, were detected by this method. These results suggest that OFQ^{1-17} is processed to smaller peptides in mouse and monkey hypothalamic neurons.

Introduction

Three major types of opioid receptors, μ, δ and κ have been cloned and characterized (Chen *et al.*, 1993; Kieffer *et al.*, 1992; Meng *et al.*, 1993). All appear to contain seven putative transmembrane (TM) domains and are coupled to Gi/Go proteins (Reisine and Bell, 1993; Uhl *et al.*, 1994). In addition to the three classical opioid receptor types an orphan "opioid-like" receptor, LC132, has also been identified. LC132 shares extensive sequence homology with the classic opioid receptors but does not bind any of the known opioid ligands with high affinity (Bunzow *et al.*, 1994). Orphanin FQ (OFQ or Nociceptin), a seventeen amino acid peptide bearing sequence homology to dynorphin A has been identified as an endogenous ligand for the LC132 receptor (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995). OFQ^{1-17} appears to be a potent agonist at the LC132 receptor as determined by its ability to inhibit forskolin-stimulated adenylyl cyclase activity (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995). The amino terminal sequence of OFQ^{1-17}, FGGF, differs only by a single hydroxyl group from the canonical amino terminal sequence YGGF of the opioid peptides. In spite of this conserved sequence, OFQ fails to bind the μ, δ or κ opioid receptors with significant affinity. This suggests that the evolutionary conservation or divergence of the amino terminus may be important in defining receptor selectivity.

The prepro(pp)OFQ cDNA has been cloned and its sequence indicates that the peptide OFQ^{1-17} is contained within a larger precursor protein (Mollereau *et al.*, 1996a; Nothacker *et al.*, 1996) (Fig. 1). The precursor contains a series of hydrophobic amino acids at its amino terminus that may constitute a signal sequence, a motif which is conserved among the opioid peptide precursors. The OFQ^{1-17} peptide is flanked by paired basic amino acids, a proteolytic cleavage motif found in the majority of prohormones (Seidah and Chretien, 1992; Thomas *et al.*, 1988). Downstream of OFQ^{1-17} are additional

Figure 1

Mouse	M K I L F C D V L L L S L L S S V F S S C P R D C L T C Q E K L
Rat	M K I L F C D V L L L S L L S S V F S S C P E D C L T C Q E R L
Human	M K V L L C D L L L L S L F S S V F S S C Q R D C L T C Q E K L

Mouse	H P A P D S F N L K T C I L Q C E E K V F P R P L W T V C T K V M
Rat	H P A P G S F N L K L C I L Q C E E K V F P R P L W T L C T K A M
Human	H P A L D S F D L E V C I L E C E E K V F P S P L W T P C T K V M

Mouse	A S G S G Q L S P A D P E L V S A A L Y Q P K A S E M Q H L [K R]
Rat	A S D S E Q L S P A D P E L T S A A L Y Q S K A S E M Q H L [K R]
Human	A R S S W Q L S P A A P E H V A A A L Y Q P R A S E M Q H L [R R]

Mouse	M P R V R S L V Q V R D A E P G A D A E P G A D A E P G A D D A
Rat	M P R V R S V V Q A R D A E P E A - - - - - - D A E P V A D E A
Human	M P R V R S L F Q E Q E E - P E P - - - - - - - - - - - G M E E A

Mouse	E E V E Q K Q L Q [K R] F G G F T G A R K S A R K L A N Q [K R] F
Rat	D E V E Q K Q L Q [K R] F G G F T G A R K S A R K L A N Q [K R] F
Human	G E M E Q K Q L Q [K R] F G G F T G A R K S A R K L A N Q [K R] F

Mouse	S E F M R Q Y L V L S M Q S S Q [R R R] T L H Q N G N V	187
Rat	S E F M R Q Y L V L S M Q S S Q [R R R] T L H Q N G N V	181
Human	S E F M R Q Y L V L S M Q S S Q [R R R] T L H Q N G N V	176

Figure 1. The deduced amino acid sequences of the mouse, rat and human ppOFQ. Potential cleavage sites are boxed. The OFQ^{1-17} peptide is underlined. Closed circles denote amino identity between species.

paired basic amino acids that flank two potentially bioactive peptides (Mollereau *et al.*, 1996a; Nothacker *et al.*, 1996). The amino acid sequence of all three of these peptides is identical in human, mouse and rat suggesting that they are important bioactive neuropeptides conserved across species (Mollereau *et al.*, 1996a; Nothacker *et al.*, 1996) (Fig. 1). The postulated bioactivities of OFQ include anti-opioid effects and systemic vasorelaxant properties (Gumusel *et al.*, 1997; Mogil *et al.*, 1996b). The discovery of a naturally occurring anti-opioid peptide suggests the possibility of designing novel analgesic compounds.

Here we report the detection of multiple forms of OFQ^{1-17}- containing peptides in rodent and monkey hypothalamic extracts by reversed-phase high performance liquid chromatography (RP-HPLC) and radioimmunoassay (RIA). OFQ^{1-17} was the major form of OFQ immunoreactivity found in hypothalamic extracts of all species examined. We have also demonstrated a limited distribution of OFQ immunoreactivity in different mouse tissues. In addition, the molecular weights of OFQ^{1-17} containing peptides produced in mouse hypothalamic extracts have been estimated by SDS-polyacrylamide tube gel electrophoresis and appear to correspond to: the full-length precursor (~24-30 kD), a biosynthetic intermediate (~7-9 kD), the OFQ^{1-17} peptide (1.8 kD) and a form of OFQ immunoreactivity with an apparent molecular weight smaller than 1.8 kD. Finally, we have demonstrated by in situ hybridization, that ppOFQ mRNA is expressed in the hypothalamic region used in these studies of OFQ peptide processing.

Materials and Methods

Antisera Production

Synthetic OFQ^{1-17} peptide was obtained from Phoenix Pharmaceuticals. OFQ^{1-17} was coupled to BSA in a molar ratio of 10:1 using the carbodiimide method. The resulting conjugate was lyophilized and sent to a commercial vendor (Covance) for rabbit immunization.

Radioimmunoassay (RIA)

Tyr^{14} OFQ^{1-17} was obtained from Phoenix Pharmaceuticals and iodinated by the chloramine T method (Allen *et al.*, 1978, 1988). The reaction mixture was fractionated over a G-10 column in 10% acetic acid and the peak of radioactivity was collected manually (6-7 drops), diluted with deionized water containing 30 µl Aprotinin (Sigma) and used as the tracer in the RIA. Synthetic OFQ^{1-17} was also obtained from Phoenix Pharmaceuticals and used to generate all standard curves. All basic RIA procedures have been described previously (Allen *et al.*, 1978,1988). A representative standard curve is shown in Figure 2.

Tissue Extraction

Tissue samples prepared from individual animals, or pooled tissues where indicated, were homogenized in 10% acetic acid, containing 0.5 mg/ml bovine serum albumin (BSA) and 3 mM phenylmethyl sulfonyl fluoride, frozen and thawed three times, centrifuged, and the resulting supernatants were frozen and lyophilized. For the RIA, samples were resuspended in phosphate buffer containing β-mercaptoethanol and BSA as previously described (Allen *et al.*, 1978,1988).

Figure 2

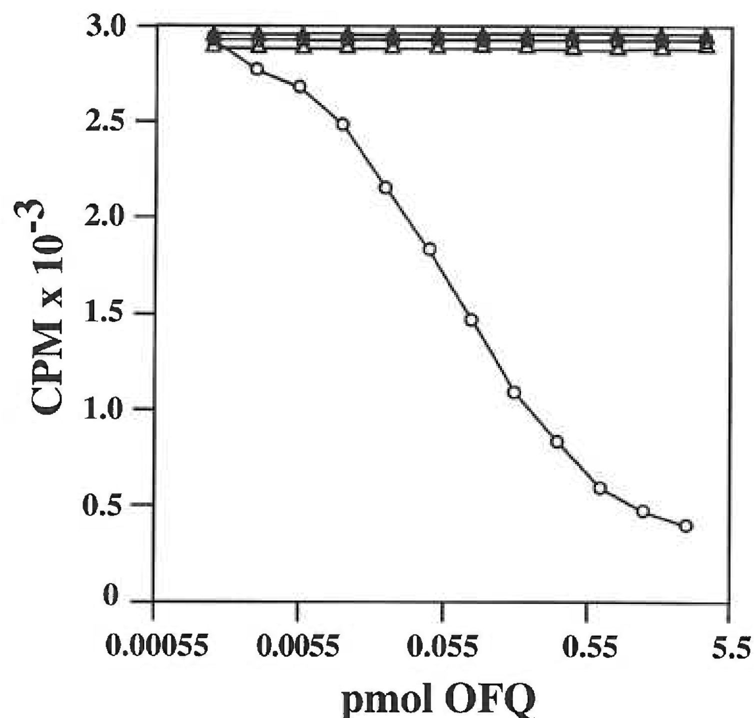

Figure 2. A representative standard curve of the OFQ radioimmunoassay. Twelve, 2-fold dilutions of synthetic OFQ^{1-17} standard in the amounts shown were incubated for 16 h at 4° C with the antiOFQ^{1-17} antiserum (Rb49 ;1:12,000 dilution) and ^{125}I Tyr^{14} OFQ as tracer. Bound CPM were separated from free as previously described (Allen *et al.*, 1978). The closed triangles are Dynorphin A (Peninsula Laboratories). The closed squares are 2-fold dilutions of mouse whole pituitary extracts. The equivalent of 1/10 (approximately 70 pmol of total β-endorphin immunoreactivity) of one mouse pituitary was added to the first tube of the dilution series. This extract contains all known forms of β-endorphin, i.e. acetylated and carboxy-shortened, and β-lipotropin (Allen *et al.*, 1988). The open triangles are OFQ^{1-7}. The open squares are OFQ^{1-11}. Thus, the Rb49 anti-OFQ antiserum does not crossreact with dynorphins or endorphins and is directed at the carboxy terminus of OFQ^{1-17}. The cross-reactivities of OFQ^{1-7}, pituitary extracts and dynorphin A were determined in separate assays and exhibited no immunoreactivity.

Reversed-phase high performance liquid chromatography (RP-HPLC)

For RP-HPLC, supernatants from 15 rat or mouse hypothalami were pooled following centrifugation, frozen and lyophilized. The pre-optic area (POA~70mg) from a Rhesus monkey hypothalamus was dissected and processed identically. The samples shown in Figure 3 B, C and D were centrifuged through a 30 kD molecular weight cut-off spin column (Microcan). Following lyophilization, samples were resuspended in 1 ml of 0.1% trifluoroacetic acid (TFA) and injected onto a Vydac (214TP54) RP-HPLC column (C4, 300 angstrom pore size; The Separations Group, Hesperia, CA). The samples shown in 3C and D were injected 0.1% TFA containing 5% acetonitrile. The extracts were fractionated using a Waters® (Milford, NJ) RP-HPLC system with a linear gradient of 0-40% or 5-36% acetonitrile in 0.1% TFA and a flow rate of 1 ml/min. Aliquots of the resulting fractions were frozen and lyophilized.

SDS-Polyacrylamide Gel Electrophoresis

Hypothalamic extracts from 10 mice were resuspended in gel loading buffer and resolved by polyacrylamide tube gel electrophoresis. Tube gels of 8 mm inner diameter, consisted of 12.5% polyacrylamide with an acrylamide to bis-acrylamide ratio of 40:1 and run at 125 volts for 3 h in Tris-acetate buffer, containing 0.1% SDS. Molecular weight standards were: ovalbumin, MW 42 kD; carbonic anhydrase, MW 27 kD; β lactoglobulin, MW 18.9 kD; lysozyme, MW 14 kD; bovine trypsin inhibitor, 6 kD (BioRad); and alpha-N-acetyl-β-endorphin $^{1-27}$ (Peninsula) MW 3.1 kD and OFQ$^{1-17}$, approximately 1.8 kD (Phoenix Pharmaceuticals). For RIA, 2 mm gel slices were eluted overnight in 0.25 ml of phosphate buffer (pH 7.6) containing 0.01% SDS and 0.04% Triton-X.

Animals

Twenty to 30g male and female mice (C57Bl/6J x 129/sv) and juvenile Wistar rats (18-19 D) were used in these studies. Rats were anesthetized with halothane and animals were sacrificed by cervical dislocation. Hypothalamic tissue was obtained from a female Rhesus macaque (age; 7 y, 4 d), #M359 Oregon Regional Primate Research Center #305-16087. All tissues were dissected and stored on dry ice immediately and stored at -80° C until extracted. All procedures described in the present study were performed in accordance with institutional guidelines based on NIH recommendations.

In situ hybridization

A cDNA clone containing ppOFQ was generated by RT-PCR using total RNA from a single mouse hypothalamus. Oligonucleotide primers were chosen to generate the full-length cDNA based on the sequence available in GeneBank. From this construct, a 244 base pair PstI fragment, including the coding region for the mature OFQ peptide, was subcloned into Bluescript (Stratagene) such that an ^{35}S-labeled antisense riboprobe was generated using T3 polymerase (BRL). Control sense riboprobes were generated from a linearized vector using T7 polymerase (BRL). Hybridization was performed on slide-mounted 20 micron sections prepared from paraformaldehyde-perfused adult male mouse brains as previously described (Bunzow *et al.*, 1994). The slides were first exposed to β-Max film for 2 days then subsequently dipped in NBT-2 Kodak emulsion and exposed for 2 weeks.

Statistical analyses

All statistical analyses were carried out using the Statview™ for Macintosh software.

Results

Distribution of OFQ in selected mouse tissues

Initial observations using histochemical methods indicated a wide distribution of the OFQ receptor in the rodent brain with some of the most intense signals found in the hypothalamus (Anton *et al.*, 1996). Of the tissues we examined, the hypothalamus and spinal cord contained the highest levels of OFQ immunoreactivity. Hypothalamic levels of OFQ immunoreactivity were 10.1+/-1.6 pmol/g (n=6). This is equivalent to approximately 0.2+/-0.026 pmol per hypothalamic tissue block analyzed. Extracts of spinal cord contained 0.05+/- 0.012 pmol of OFQ immunoreactivity per two cm sections of spinal cord. OFQ immunoreactivity was below detectable levels in extracts of cardiac muscle, pituitary, striatum, cerebellum and locus coeruleus. For comparison, β-endorphin levels were very similar to OFQ levels in the mouse hypothalamus and spinal cord (0.15+/-0.04 pmol/hypothalamus and 0.19+/- 0.07 pmol/ per two cm sections, respectively). These results demonstrated that the hypothalamus was a rich source of OFQ immunoreactivity and could be used to study OFQ processing.

RP-HPLC of rat , mouse and monkey hypothalamic extracts

In initial studies we utilized RP-HPLC to fractionate the forms of OFQ immunoreactivity produced in rodent hypothalamic neurons. Extracts of hypothalamic tissue from either rat or mouse were pooled, fractionated by RP-HPLC and the resulting fractions were assayed for OFQ immunoreactivity by RIA. The major peak of OFQ immunoreactivity observed in the mouse hypothalamic extracts eluted at 36 minutes which coincides with the elution time of synthetic OFQ^{1-17} (Fig. 3A). The small doublet of immunoreactivity eluting between 60 and 63 minutes most likely represents the ppOFQ. The major peak of immunoreactivity in rat hypothalamic extracts also eluted at 36 min (Fig. 3B). We observed no significant immunoreactivity eluting at 60-63 minutes in rat

Figure 3

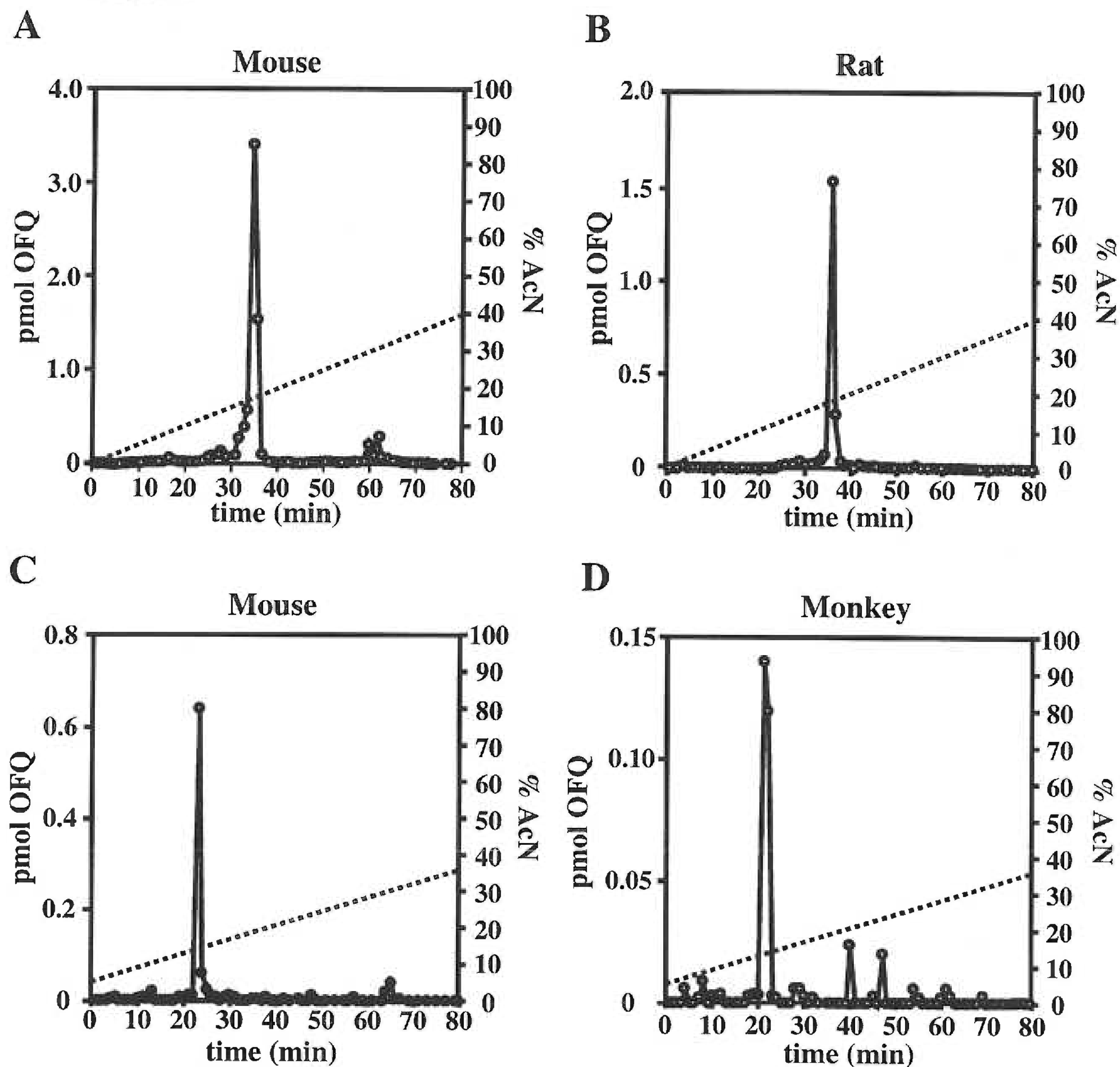

Figure 3. RP-HPLC fractionation of mouse, rat and monkey hypothalamic extracts. RP-HPLC was performed using a C4 column (Vydac) and a gradient of 0-40% acetonitrile in 0.1% trifluoroacetic acid (TFA) (A and B). In the fractionations shown in C and D the gradient was 5-36% acetonitrile in 0.1% TFA. A) mouse hypothalamus; B) rat hypothalamus; C) mouse hypothalamus; D) monkey hypothalamus (pre-optic area) Aliquots of the fractions were lyophilized and assayed for OFQ immunoreactivity. Synthetic OFQ^{1-17} elutes at 36 min in the gradient shown in panels A and B. Synthetic OFQ^{1-17} elutes at 22 min and OFQ^{1-11} elutes at 10 in the gradient shown in panels C and D. Panels A and B are examples of replicate determinations and C and D single determinations.

hypothalamic extracts but did observe a small shoulder of OFQ immunoreactivity in mouse hypothalamic extracts that eluted immediately prior to the major peak of OFQ^{1-17}.

In the initial studies we used a gradient from 0 to 40% acetonitrile in 80 min to fractionate OFQ containing peptides. In the next set of fractionations we flattened the gradient to attempt to resolve other peaks of immunoactivity potentially co-eluting with OFQ^{1-17} . Figure 3C shows that no additional peaks of OFQ immunoreactivity were resolved in mouse hypothalamic extracts by using a gradient from 5 to 36% acetonitrile in 80 min. Again, the major peak of OFQ immunoreactivity found in mouse extracts co-eluted with synthetic OFQ^{1-17} (22 min). We also found a peak of OFQ immunoreactivity eluting at about the same percentage acetonitrile (64-65 min) as in the initial gradient. In contrast to rodent hypothalamic extracts, monkey hypothalamic tissue contained multiple peaks of OFQ immunoreactivity. Minor peaks were found at 4, 8, 28-29, 40, 47, 54 and 61 min. The major peak of immunoactivity eluted at 21-22 min coincident with synthetic OFQ^{1-17} (Fig. 3D). Figure 3C and D also show a small peak of immunoreactivity eluting at 9-12 minutes. This coincides with the elution position of synthetic OFQ^{1-11}. The antiserum used in this study recognizes OFQ^{1-11} 40 to 50-fold less than the parent peptide OFQ^{1-17}.

SDS-PAGE of mouse hypothalamic extracts

In order to determine whether any OFQ-containing peptides were co-eluting with the parent peptide (OFQ^{1-17}) we fractionated mouse hypothalamic extracts on SDS-PAGE tube gels. Following electrophoresis, the gel slices were eluted and the eluates assayed individually for OFQ immunoreactivity. Using this approach we found OFQ-containing molecules that migrated with apparent molecular weights of 24-30 kD; which probably corresponds to post-translationally modified forms of ppOFQ (Fig. 4). We also identified

Figure 4

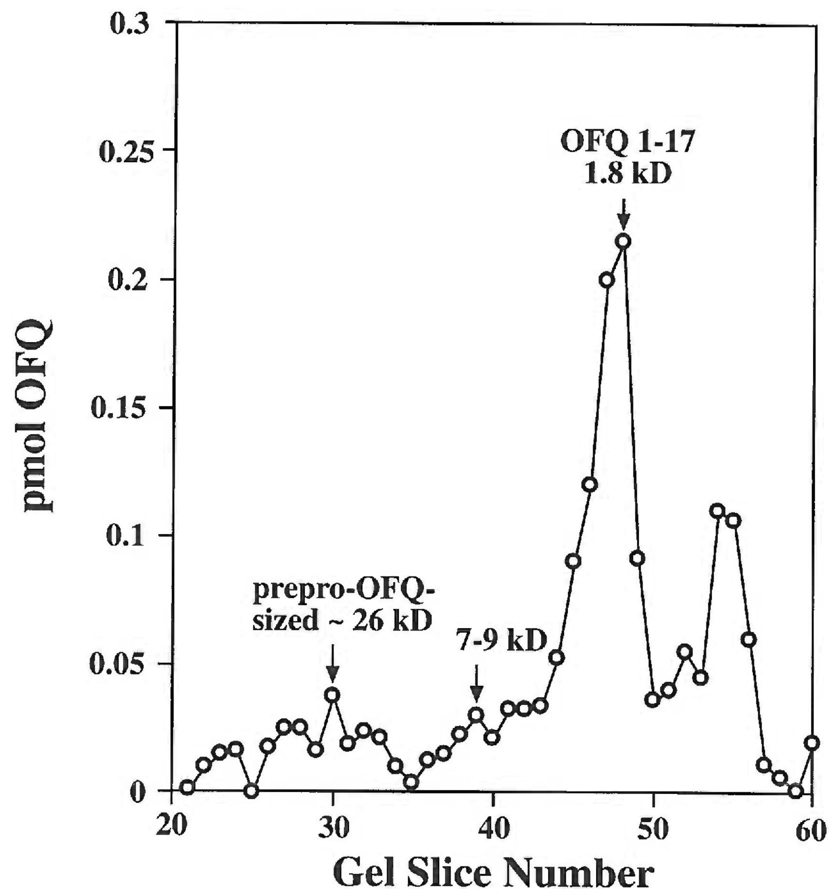

Figure 4. SDS-PAGE of mouse hypothalamic extracts. Mouse hypothalamic extracts were fractionated on a 12.5% 120 mm poly-acrylamide tube gel. The gel was then cut into 2 mm slices and the slices eluted for 16 h. The eluates were assayed for OFQ immunoactivity as described in the methods. Apparent molecular weights were determined by co-electrophoresing commercial standards (BioRad) and synthetic OFQ^{1-17}. Recovery was greater than 90% of input immunoreactivity. The results are representative of two separate determinations.

OFQ-containing molecules that migrated at 7-9 kD, which may represent biosynthetic intermediates containing the OFQ peptide. The fully processed 17 amino acid peptide was also detected and found to co-migrate with synthetic OFQ^{1-17} (1.8 kD). Additionally, we identified a peak of OFQ immunoreactivity with a molecular weight smaller than OFQ^{1-17} (~1 kD).

Localization of ppOFQ mRNA in the mouse hypothalamus by in situ hybridization

To determine the sites of ppOFQ synthesis in the hypothalamus an in situ hybridization study was performed. Although transcripts for ppOFQ were expressed diffusely throughout the hypothalamus, particularly dense signals were detected in the arcuate nucleus (ARC) (Fig. 5). Control experiments in which a sense ppOFQ riboprobe was used failed to show any hybridization signal (data not shown). Thus, the sites of ppOFQ mRNA synthesis coincide well with detection of the peptide in the hypothalamic tissue blocks we analyzed by RIA.

Discussion

The data presented here is the first quantification of OFQ immunoreactivity in the rodent and sub-human primate hypothalamus. The results of the present studies are consistent with the notion that the major immunoreactive OFQ containing peptide in the hypothalamus is OFQ1-17. Recent quantitative studies using RP-HPLC and RIA have shown that the major form of OFQ in the ventral spinal cord of the rat co-elutes with OFQ1-17 (Schuligoi et al., 1997). These studies also detected minor forms of OFQ immunoreactivity eluting later than OFQ1-17, which is consistent with our findings. These forms were not apparent in our fractionation of mouse and rat hypothalamus. Further,

Figure 5

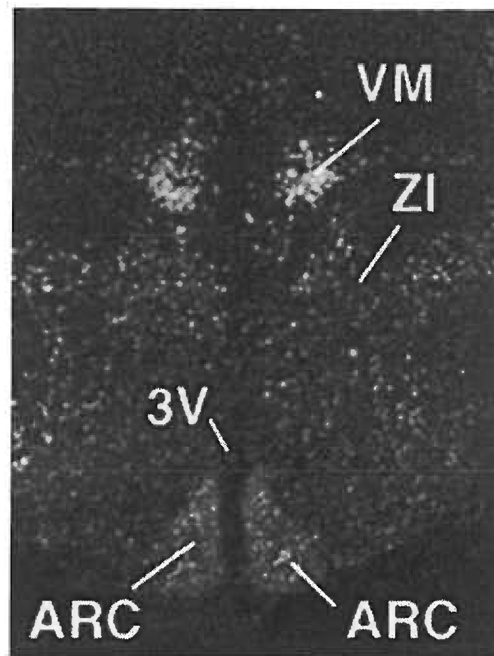

Figure 5. In situ hybridization detection of ppOFQ mRNA in the mouse hypothalamus. 20 micron sections were hybridized to a 244 base-pair antisense ^{35}S-labeled riboprobe, washed and then processed as previously described (Bunzow *et al.*, 1994). Significant labeling was detected in the ARC (Arcuate Nucleus). 3V designates the Third Ventricle and ZI (Zona Incerta).

those studies and the studies presented here, detected OFQ immunoreactivity eluting before OFQ1-17. We also found immunoreactive material eluting late in the gradient (61-65 min) in mouse and monkey, but not in rat hypothalamic extracts. The detection of multiple forms of OFQ immunoreactivity suggests that OFQ1-17 is processed through series of biosynthetic intermediates as are the opioid peptides.

Studies with synthetic peptides have demonstrated that our OFQ antiserum is directed against the carboxy terminus of OFQ1-17, as it fails to interact with OFQ1-7 and exhibits only slight cross-reactivity with OFQ1-11 (Fig. 2). The studies quantifying OFQ immunoreactivity in the rat spinal cord did not report crossreactivity of their antiserum, thus it is difficult to interpret which OFQ-containing moieties they detected.

The OFQ immunoreactive material migrating with an apparent molecular weight smaller than OFQ1-17 in the SDS-PAGE analysis must contain mainly the C-terminal region of the peptide. The finding that OFQ1-17 was apparently processed to a smaller form has some interesting implications. A striking feature of OFQ1-17 is the presence of two RK sites in its primary amino acid sequence (Fig.1). Paired, basic amino acids such as these often serve as cleavage sites for the prohormone convertases involved in precursor processing. However, the juxtaposition of RK rather that KR is used less frequently as a cleavage motif (Seidah and Chretien, 1992). The OFQ immunoreactivity eluting at 10 min in figure 3D co-elutes with synthetic OFQ1-11. The cross-reactivity studies shown here indicate that OFQ1-11 is approximately 40 times less potent than OFQ1-17 in our RIA. This suggests that if the material eluting at 10 min in the monkey hypothalamic extracts is indeed OFQ1-11 there is much more of it than is apparent the RP-HPLC profile.

Of interest here is recent work showing that OFQ1-17 can be catabolized by Aminopeptidase N and Endopeptidase 24.15 to several products (Montiel et al., 1997). A major degradation product was OFQ1-11, which would be generated by cleavage at the RK site at amino acid positions 12 and 13. The immunoreactive material migrating with an

apparent molecular weight of 1 kD in the SDS-PAGE analysis of mouse hypothalamic extracts would be the size predicted for a cleavage liberating OFQ10-17. This product would be generated by a cleavage at the RK site at amino acid positions 8 and 9.

Recent structure-activity studies performed on OFQ-derived peptides have shown that OFQ1-7 and OFQ1-11 will inhibit forskolin-stimulated cAMP production in CHO cells at very high concentrations. Also, both of these truncated forms of OFQ, as well as OFQ1-5 , will bind the OFQ receptor, albeit with 100-1000 fold less affinity (Reinscheid et al., 1996). Therefore, receptor activation by OFQ depends on the carboxy extensions of the molecule suggesting that OFQ may interact with its receptor via different configurations than do the opioid peptides. OFQ11-17 will not inhibit forskolin-stimulated cAMP production, but will bind the OFQ receptor with less than micromolar affinity (Reinscheid et al., 1996). The observation that OFQ is processed to smaller forms in the hypothalamus suggests that either there is an additional OFQ receptor subtype that binds these ligands or CHO cells do not express all of the transduction machinery necessary for the truncated OFQ peptides to functionally couple the receptor to second-messenger systems. Another possibility is that the smaller OFQ-containing moieties are natural degradation products of OFQ1-17. Immunocytochemical studies have demonstrated both OFQ-containing cell bodies and fibers in several hypothalamic areas (Riedl et al., 1996). Therefore, the OFQ peptide profiles shown here represent the sum of OFQ peptides contained in those neuronal compartments.

A growing body of evidence suggests that OFQ is an important neuropeptide (Anton et al., 1996; Uhl et al., 1994; Vaughan et al., 1997). Recent electrophysiological studies have shown OFQ to be a potent modulator of potassium conductances in the rat periaqueductal gray (Vaughan et al., 1997) and the arcuate nucleus of the mediobasal hypothalamus. In the case of the hypothalamus, it has been determined that OFQ receptors are expressed by β-endorphin neurons in the hypothalamus and that OFQ hyperpolarizes

these cells (M. Kelly, personal communication). These findings suggest that there may be an intricate interplay between the opioid peptides and OFQ in modulating a variety of biological processes including analgesia, stress, reproduction and various autonomic functions (Vaughan *et al.*, 1997).

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Chapter 2

Orphanin-FQ/Nociceptin (OFQ/N) Modulates the Activity of Suprachiasmatic Nucleus Neurons

Orphanin-FQ/Nociceptin (OFQ/N) Modulates the Activity of Suprachiasmatic Nucleus Neurons

Charles N. Allen[1,2], Zhi-Gen Jiang[1], Koji Teshima[1], Tristan Darland[3], Masayuki Ikeda[5], Cole S. Nelson[1], Denise I. Quigley[4], Tohru Yoshioka[6], Richard G. Allen[1], Michael A. Rea[6], and David K. Grandy[2,3]

[1]Center for Research on Environmental and Occupational Toxicology, [2] Dept. of Physiology and Pharmacology, [3]Dept. of Cell and Developmental Biology, [4] Dept. of Molecular and Medical Genetics, Oregon Health Sciences University, 3181 S.W. Sam Jackson Park Road, Portland, Oregon 97201-3098, USA. [5]Department of Molecular Neurobiology, School of Human Sciences, Waseda University, 3-579-15 Mikajima, Tokorozawa, Saitama 359, Japan [6]Biological Rhythms and Integrative Neurosciences Research Institute, Air Force Research Laboratory, Brooks AFB, Texas 78235, USA.

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Corresponding Author:
Charles N. Allen
CROET, L606
Oregon Health Sciences University
3181 S.W. Sam Jackson Park Road
Portland, Oregon 97201-3098
phone: (503) 494-2507
fax: (503) 494-6831
email: allenc@ohsu.edu

Abbreviated title: Orphanin-FQ and the Suprachiasmatic Nucleus

Abstract

Neurons in the suprachiasmatic nucleus (SCN) constitute the principal circadian pacemaker of mammals. In situ hybridization studies revealed expression of orphanin FQ/nociceptin (OFQ/N) receptor (NOR) mRNA in the SCN while no expression of mRNA for preproOFQ/N (ppOFQ/N) was detected. The presence of OFQ/N peptide in the SCN was demonstrated by radioimmunoassay. SCN neurons (88%) responded dose-dependently to OFQ/N with an outward current (EC50 = 22.1 nM) that was reduced in amplitude by membrane hyperpolarization and reversed polarity near the theoretical potassium equilibrium potential. $[Phe^1\psi(CH_2\text{-}NH)Gly^2]OFQ/N(1\text{-}13)NH_2$ (3 μM), a putative NOR antagonist, activated a small outward current and significantly reduced the amplitude of the OFQ/N-stimulated current. OFQ/N reduced the NMDA receptor-mediated increase in intracellular Ca^{2+}._ When injected unilaterally into the SCN of Syrian hamsters housed in constant darkness OFQ/N (1-50 pmol) failed to alter the timing of hamster's wheel running activity. However, injection of OFQ/N (0.1 - 50 pmol) prior to a brief exposure to light during the mid-subjective night significantly attenuated the light-induced phase advances of the activity rhythm. These data are consistent with the interpretation that OFQ/N acting at specific receptors modulates the activity of SCN neurons and, thereby, the response of the circadian clock to light.

Key words Orphanin-FQ, nociceptin, suprachiasmatic nucleus, circadian rhythm, potassium current, calcium

Introduction

Organisms display regular, daily fluctuations in behavioral and physiological processes called circadian rhythms. In mammals, the principal pacemaker of circadian rhythms is located in the suprachiasmatic nucleus (SCN), a paired structure lying dorsal to the optic chiasm in the ventral hypothalamus (Meijer and Rietveld, 1989). SCN-driven circadian rhythms are synchronized to the environmental light-dark (LD) cycle through a process called "photic entrainment", whereby the phase and period of the circadian clock are adjusted by exposure to ambient light maintaining the proper phase relationships between circadian rhythms and relevant daily environmental changes (DeCoursey, 1964; Morin, 1994). Photic information is transmitted directly to the SCN via the glutamatergic retinohypothalamic tract (RHT) (De Vries *et al.*, 1993; Moore *et al.*, 1995; Morin, 1994). Neurons in the retina project directly to the SCN, use glutamate as a neurotransmitter, and activate NMDA and Ampa receptors (Kim and Dudek, 1991; Rea *et al.*, 1993; Jiang *et al.*, 1997). Activation of NMDA receptors can phase advance or phase delay the biological clock depending on the circadian time of application (Ding *et al.*, 1994; Shirakawa and Moore, 1994). A signaling pathway has been proposed in which activation of NMDA receptors increases intracellular Ca^{2+}, which in turn activates nitric oxide synthase and increases nitric oxide that, via steps involving the activation of protein kinase G and ryanodine receptors alters the timing of the circadian clock (Ding *et al.*, 1994, 1998; Weber *et al.*, 1995a,b).

The SCN contains a number of peptide neurotransmitters including vasopressin, vasoactive intestinal peptide (VIP) and neuropeptide Y (Van den Pol and Tsujimoto, 1985). These peptides are believed to be important regulators of SCN neuronal activity and the phase of the circadian clock (Inouye, 1996). Recently a G-protein coupled receptor that is 65% identical to the μ-, δ- and κ-opioid receptors was discovered. This orphan receptor shows no high-affinity binding to selective opioid agonists or antagonists (Bunzow *et al.*,

1994; Mollereau *et al.*, 1994). The endogenous ligand for this receptor is a heptadecapeptide (FGGFTGARKSARKLANQ) that resembles dynorphin and was named both orphanin-FQ (Reinscheid *et al.*, 1995) and nociceptin (Meunier *et al.*, 1995) (OFQ/N). Synthetic 125I-OFQ/N, which has a low affinity for the μ-, δ- and κ-opioid receptors, binds the OFQ/N receptor (NOR) in a saturable and specific manner that is insensitive to the opioid antagonist naloxone (Nothacker *et al.*, 1996; Mollereau *et al.*, 1996a). OFQ/N is synthesized as part of a precursor protein, preproOFQ/N (ppOFQ/N), whose organization is similar to that of pro-opiomelanocortin (POMC), preproenkephalin (PPE) and preprodynorphin (PPD) (Nothacker *et al.*, 1996; Mollereau *et al.*, 1996a). In the course of an in situ hybridization survey of the rat hypothalamus we discovered that NOR was densely expressed in the SCN. Given the presence of NOR in the SCN we performed experiments to determine whether OFQ/N can modulate the activity of SCN neurons and alter the timing of the circadian clock.

Materials and Methods

In situ hybridization

In situ hybridization was performed using sense and antisense [^{35}S]UTP-labeled riboprobes consisting of the first 100 N-terminal amino acids of NOR which has the lowest homology to the μ-, δ- and κ-opioid receptors (Bunzow *et al.*, 1994). For detection of ppOFQ/N, a nearly full-length OFQ/N clone was prepared by reverse transcriptase PCR using oligonucleotide primers. The cDNA used to make the riboprobe was sequenced and found to be identical to the published sequence (Meunier *et al.*, 1995). Riboprobes were purified on Sephadex G-50 columns (Pharmacia, Piscataway, NJ) and diluted to a final concentration of 2 x 106 cpm/ml in a hybridization solution consisting of: 500 μg/ml tRNA, 50 μM dithiothreitol (DTT), 50% formamide, 0.25 mM NaCl, 1X Denhardt's solution and

10% dextran sulfate. Adult male Spraque-Dawley rats were anesthetized with isoflurane and perfused with 4% paraformaldehyde dissolved in borate buffer, pH 9.5. The brain was dissected out and incubated overnight in fix plus 20% sucrose. Twenty-micron thick sections were cut on a cryostat and mounted onto Superfrost Plus slides (VWR, San Francisco, CA). The slides were fixed in 4% paraformaldehyde dissolved in PBS, permeabilized with proteinase K, acetylated in acetic anhydride and triethanolamine, and dehydrated in ethanol. The probe containing solution was placed on the slides and incubated overnight. The slides were rinsed with 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 dehydrated in ascending concentration of ethanol. The slides were exposed to β-max film for 2-3 d before being dipped in NBT-2 emulsion (Kodak, Rochester, NY). After 2 weeks of exposure at 4°C, the slides were developed in D-19 developer (Kodak) and counterstained with thionin. Alternating slides were used to conduct the same _survey with a sense riboprobe and with thionin staining alone. The sections were mounted on glass slides, exposed to Cronex film (Dupont, Wilmington, DE) for 5 days, dipped in emulsion and exposed for 2 weeks.

Preparation of SCN brain slices

Male Sprague-Dawley rats (200-300 gm) were maintained on a light/dark schedule of 12 hr light and 12 hr dark (LD 12:12; lights on 8:00 A.M.) for at least 2 weeks. During the lights on phase rats were deeply anesthetized with halothane and their brains removed and placed in ice-cold Krebs solution consisting of (in mM): NaCl, 126; KCl, 2.5; NaH_2PO_4, 1.2; $MgCl_2$, 1.2; $CaCl_2$, 2.4; glucose, 11; $NaHCO_3$, 26; saturated with 95% O_2 and 5% CO_2. Horizontal (500-μm-thick) or coronal (300-μm-thick) slices of hypothalamus were cut with a vibratome. The horizontal slices were secured in the recording chamber and completely immersed in continuously flowing, warmed (36°C) Krebs' solution with the $NaHCO_3$ reduced to 20 mM, pH 7.4. The SCN was identified in the slice, by the used of a

stereomicroscope, as the gray matter region immediately dorsal to the optic chiasm and within 500 mm of the midline. Additional experiments were performed with the SCN visualized using Infrared Differential Interference Videomicroscopy (IR-DIC) (Dodt and Zieglg®nsberger, 1990). Coronal slices (300 μm) were mounted on the stage of a Optiphot-2 microscope (Nikon, Japan) and visualized with a IR-CCD camera and camera controller (Hamamatsu, Japan). The University Animal Care Committee approved all procedures involving animals.

Patch clamp recording

Recordings were made using the whole cell and perforated patch-clamp modes from 0.5 to 12 hr after preparation of the slices. Whole-cell patch electrodes had outside tip diameters of ~1 μm and resistances of ~5 MΩ when filled with a solution containing (in mM): K^+ gluconate, 125; NaCl, 15; $CaCl_2$, 1; $MgCl_2$, 2; HEPES, 10; EGTA 11; ATP, 3; GTP, 0.3; at pH 7.3. The electrode was advanced into the brain slice and a seal with the cell membrane obtained by applying negative pressure. _Seal resistances ranged from 5 to 20 GΩ. The membrane was ruptured by further negative pressure, producing intracellular access with series resistances of 8-20 MΩ. Membrane potentials or currents were measured with n axopatch-1D amplifier (Axon Instruments, Foster City, CA), recorded on a pen recorder and an on-line personal computer (IBM AT) equipped with pClamp 5.0 (Axon Instruments).

Additional experiments were performed using the nystatin perforated patch technique (Akaike and Harata, 1994). Nystatin was dissolved in methanol (10 mg/ml), then diluted just before recording to a final concentration of 150-300 μg/ml in an electrode filling solution consisting of (in mM) 150 KCl, 10 HEPES at pH 7.2. Microelectrodes with resistances of 6-10 MΩ were pulled from borosilicate glass (WPI) and polished with a microforge (Narishige, Tokyo, Japan). Recording began 10-15 min after formation of a GΩ seal when the series resistance stabilized between 30 and 50 MΩ. The data were recorded

with a Axopatch 200A amplifier (Axon Instruments, Foster City, CA) and an on-line Macintosh G3 computer using Pulse and PulseFit (HEKA).

OFQ/N (300 nM) was ejected by pressure (2-3 psi) through a micropipette (with tip diameters of about 2 μm and 30-50 μm away from the cell body) with a Picospritizer (General Valve Corp, Fairfield, NJ) under computer control. OFQ/N antagonist and naloxone were applied by bath perfusion. Bicuculline (10 μM) and tetrodotoxin (1 μM) were routinely added to the medium to suppress spontaneous inhibitory synaptic currents, and sodium currents.

Calcium imaging

Coronal slices (210 μm) of the hypothalamus were prepared from 3 to 4 week old C57BL/6J Black mice. The slices were incubated for 1 hr in a Krebs' solution containing (in mM): NaCl 138.6, KCl 3.35, $NaHCO_3$ 21, NaH_2PO_4•H_20 9.9; D-glucose, $CaCl_2$ 2.5; and $MgCl_2$, 1 and continuously bubbled with 95% O_2/5% CO_2. The slices were incubated for 1 hr in 10 μM fura-2 AM (Molecular Probes, Eugene, OR) with 0.001% cremophore EL (Sigma). The slices were then incubated in the Krebs' media for at least 30 min then transferred to the stage of an Axioplan 2 microscope (Zeiss, Thornwood, NY) equipped with a 10X water immersion objective (Olympus, Melville, NY) and perfused at 1.5 ml/min with Krebs' solution. Changes in intracellular Ca^{2+} were estimated from the ratio of emission intensities excited by consecutive pulses of light with wavelengths of 340 and 380 nm. The images were projected onto a cooled CCD camera (C4880, Hamamatsu Photonics, Japan) which was designed for real time imaging of intracellular Ca^{2+} levels. Data analysis was performed on-line by an Argus CA50 computer based software system (Hamamatsu Photonics, Japan). All techniques were performed at room temperature.

Effects of OFQ/N on Circadian Phase

Adult, male Syrian hamsters (Mesocricetus auratus) obtained from Charles River (Wilmington, MA) were maintained in our animal facility under LD 14:10 for at least two weeks prior to experimentation. Under general anesthesia (12.5 mg/kg ketamine, 20 mg/kg xylazine, and 2 mg/kg acepromazine maleate), hamsters (130 -160 g) received intracranial cannula guides (26 ga) stereotaxically aimed at the SCN (0.8 mm anterior to bregma, 1.6 mm lateral to the midline, 2.9 mm below the dura, at an angle of 10 degrees from the sagittal plane). Cannula guides were secured in place with machine screws and dental cement. After at least one week in LD 14:10 to recover from the surgery, animals were transferred to individual cages equipped with a running wheel and maintained under constant darkness for the remainder of the experiment.

Wheel-running activity was monitored continuously by an Intel 486-based computer running Dataquest III data acquisition software (Minimitter Co., Sunriver, OR) as described elsewhere (Rea *et al.*, 1993). The onset of wheel running activity, designated as circadian time (CT) 12, was used as a phase reference point for the timing of drug administration and light exposure. CT12 on the day of treatment was estimated by extrapolation of the regression line fitted to activity onsets on the five days prior to treatment. Phase shifts of the free-running activity rhythm were estimated by comparing this value with the value for CT12 obtained after back extrapolation of the regression line fitted through activity onsets on post-treatment days 4 - 9 to the day of treatment (Rea *et al.*, 1993).

After stable free-running activity rhythms were established (8-14 days), animals were removed from their cages in darkness using an infrared viewer. At specific times relative to activity onset (CT12), hamsters received intra-SCN injections (0.3 μl) of either vehicle (0.01% bovine serum albumin in a solution of (in mM): NaCl 122, KCl 3.8, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, and $CaCl_2$ 1.2) or 50 pmol of synthetic OFQ/N. Administration was achieved using a 33 gauge infusion cannula attached to a 1 μl Hamilton syringe (Rea *et al.*, 1993). The infusion cannula extended 4.4 mm beyond the tip of the

guide, to a position near the dorsolateral border of the right SCN. In a separate experiment, hamsters received intra-SCN injections (0.3 μl) of either vehicle or a single dose (0.1 - 50 pmol) of synthetic OFQ/N given 10 minutes prior to a brief light exposure (20 lux for 10 min). Injections were timed so that light exposure occurred 7 hr after the predicted activity onset (CT 19). After treatment, the hamsters were returned to darkness and wheel-running activity was monitored for an additional 10-14 days. After data collection, the location of the injection site was verified histologically by examining 100 μm-thick vibratome sections cut through the injection site. Data were analyzed by one-way ANOVA and differences between means were tested post-hoc for significance ($p<0.05$) using the Student's Newman-Keuls test.

OFQ/N radioimmunoassay

Synthetic OFQ/N peptide (Phoenix Pharmaceuticals, Mountain View, CA) was coupled in a 10:1 ratio to bovine serum albumin using the carbodiimide method and sent to a commercial vendor for rabbit immunization (Covance, Princeton, NJ). Tyr14 OFQ/N was iodinated using the chloramine T method and fractionated over a G-10 column in 10% acetic acid; the peak of radioactivity was collected and diluted with deionized water containing 30 μM of aprotinin (Sigma). Coronal brain slices (400 μm) were prepared as described above. The SCNs were punched out using a 16 ga needle and stored at -80°C. The SCNs from six rats were pooled for the radioimmunoassay (RIA). The punches were homogenized in 10% acetic acid containing 0.5 mg/ml bovine serum albumin and 3 mM phenylmethlysulfonyl fluoride, frozen and thawed three times, and centrifuged; and the resulting supernatants were frozen and lyophilized. Before the RIA, the samples were resuspended in phosphate buffer containing β-mercaptoethanol and bovine serum albumin as previously described (Quigley *et al.*, 1998).

Results

Localization of OFQ/N peptide and receptors in the SCN

In the course of our efforts to identify G-protein coupled receptors that may modulate the firing of SCN neurons, we examined the expression of NOR in the SCN of adult rats by in situ hybridization. A radiolabeled riboprobe complimentary to the first 100 nucleotides of NOR revealed considerable mRNA expression throughout the hypothalamus and the SCN (Fig. 1A). To assess whether OFQ/N peptide was present in the SCN a quantitative OFQ/N-specific RIA was used. This sensitive assay detected 3.25 ± pmol/g of the tissue wet weight (mean ± S.E.M). This value is similar to 3.21 pmol/g reported previously for determination of OFQ/N in the rat hypothalamus (Quigley *et al.*, 1998). These data demonstrate that the OFQ/N peptide and NOR are present in the SCN.

SCN neurons synthesize several neurotransmitters including vasopressin and VIP. Since OFQ/N was detected in the SCN by RIA it was of interest to determine whether these neurons expressed ppOFQ/N mRNA. For this analysis, in situ hybridization studies were performed on serial sections of adult male rat brain using a radiolabeled rat riboprobe. The ppOFQ/N probe revealed the presence of its mRNA in the zona incerta (ZI), the CA1 and CA3 pyramidal cells of the hippocampus, the granule cells of the dentate gyrus, and cells in the ventral lateral geniculate nucleus (VGL) (Fig. 1C, D). However, four attempts failed to detect ppOFQ/N mRNA in the SCN (Fig. 1B) suggesting that OFQ/N and NOR are present in the SCN but that ppOFQ/N is synthesized elsewhere in the brain.

Electrophysiological responses of SCN neurons to OFQ/N

The abundance of NOR mRNA and OFQ/N peptide in the SCN suggested that activation of NORs would alter the activity of SCN neurons. To explore this possibility OFQ/N was applied by bath superfusion or from a micropipette to SCN neurons maintained in brain slices. In 88% (45 of 51) of the SCN neurons examined OFQ/N dose-

Figure 1

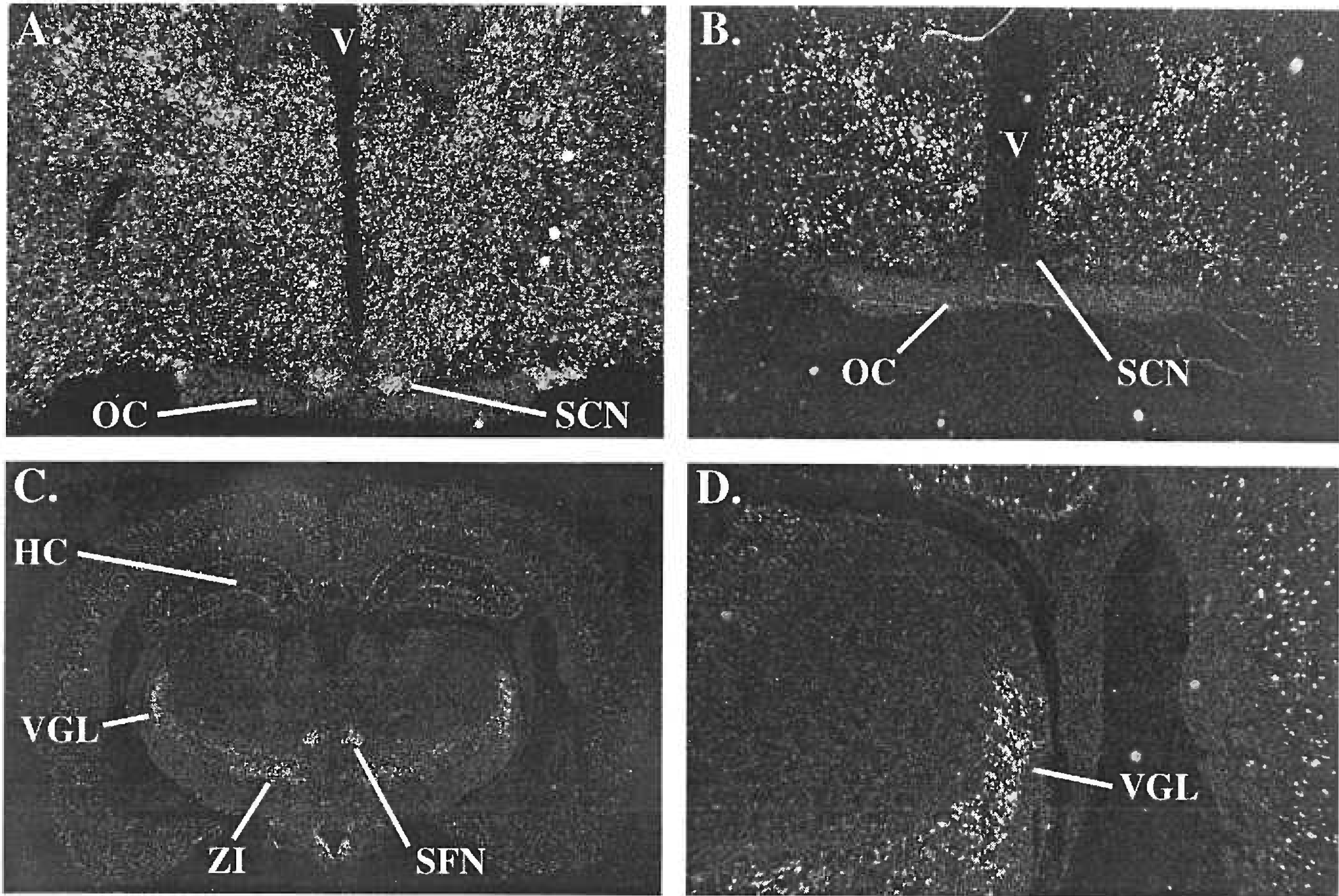


Figure 1. A. Distribution of NOR mRNA in the rat brain. Dark-field illumination of a coronal section through the level of the SCN showing dense staining within the SCN and the SON. B-D. Expression of preproOFQ/N mRNA. B. Note the lack of expression in the SCN. C. PreproOFQ/N staining in the VGL, zona incerta and the subparafasicular nucleus. D. From the same section as C showing the hybridization signal in the VGL and the region of the IGL. No signal was observed when a sense probe was used. The calibration bar is 400 μm in A, B and D and 173 μm in C. V - third ventricle, SCN - suprachiasmatic nucleus, SON - supraoptic nucleus, OC - optic chiasm, HC - hippocampus, SFN - subparafasicular nucleus, VGL - ventral lateral geniculate, ZI - zona incerta.

dependently (EC_{50} = 22.3 nM) activated a robust outward current that reached an average of 22.1 ± 4 pA (n = 11; mean ± S.E.M) at a peptide concentration of 100 nM (Fig. 2A, B). The amplitude of the OFQ/N-induced current in SCN neurons was found to be increased by membrane potential depolarization, decreased by hyperpolarization, and was always associated with an increase in input conductance (0.51 ± 0.1 nS).

To determine the ionic nature of the current induced by OFQ/N the potential at which it reversed polarity was identified. The OFQ/N-induced current reversed between -95 and -112 mV (mean -103 mV; n = 3 at a $[K^+]_o$ = 2.5 mM, Fig. 2C). This current was sensitive to changes in the extracellular K^+ concentration. Raising the $[K^+]_o$ to 10 mM shifted the reversal potential of the OFQ/N-induced current to -69 and -74 mV (n = 2).

The putative NOR antagonist $[Phe^1\psi(CH_2\text{-}NH)Gly^2]OFQ/N(1\text{-}13)NH_2$ was used to determine whether the OFQ/N activated current was mediated by its receptor or by one of the classical opioid receptors. In these experiments, 300 nM OFQ/N was applied to SCN neurons for 2 sec, and recordings were made using the nystatin perforated patch technique. Bath application of the putative OFQ/N antagonist (3 μM) resulted in a 70% inhibition of the OFQ/N-activated current (11.5 ± 2.2 pA versus 3.5 ± 0.9 pA; mean ± S.E., n = 5; Fig. 3). In contrast, naloxone had no effect on the amplitude of OFQ/N-activated currents (10.4 ± 2.1 pA versus 10.9 ± 0.8 pA, n = 3) (Fig. 3). Application of the putative OFQ/N antagonist (3 μM) alone produced a small outward current (4.6 - 20.6 pA, 6 of 9 cells) suggesting that $[Phe^1\psi(CH_2\text{-}NH)Gly^2]OFQ/N(1\text{-}13)NH_2$ has both agonist and antagonist activity at NOR. Multiple applications of OFQ/N (300 nM; 2 sec) at 1 min intervals continued to activate additional outward current. This demonstrates that the currents activated by OFQ/N antagonist application did not reduce the amplitude of OFQ/N-activated currents by maximally stimulating the OFQ/N-activated currents. Therefore, the block of the OFQ/N-activated current by $[Phe^1\psi(CH_2\text{-}NH)Gly^2]OFQ/N(1\text{-}13)NH_2$ was not caused by a saturation of NOR stimulation. These data demonstrate that OFQ/N

Figure 2

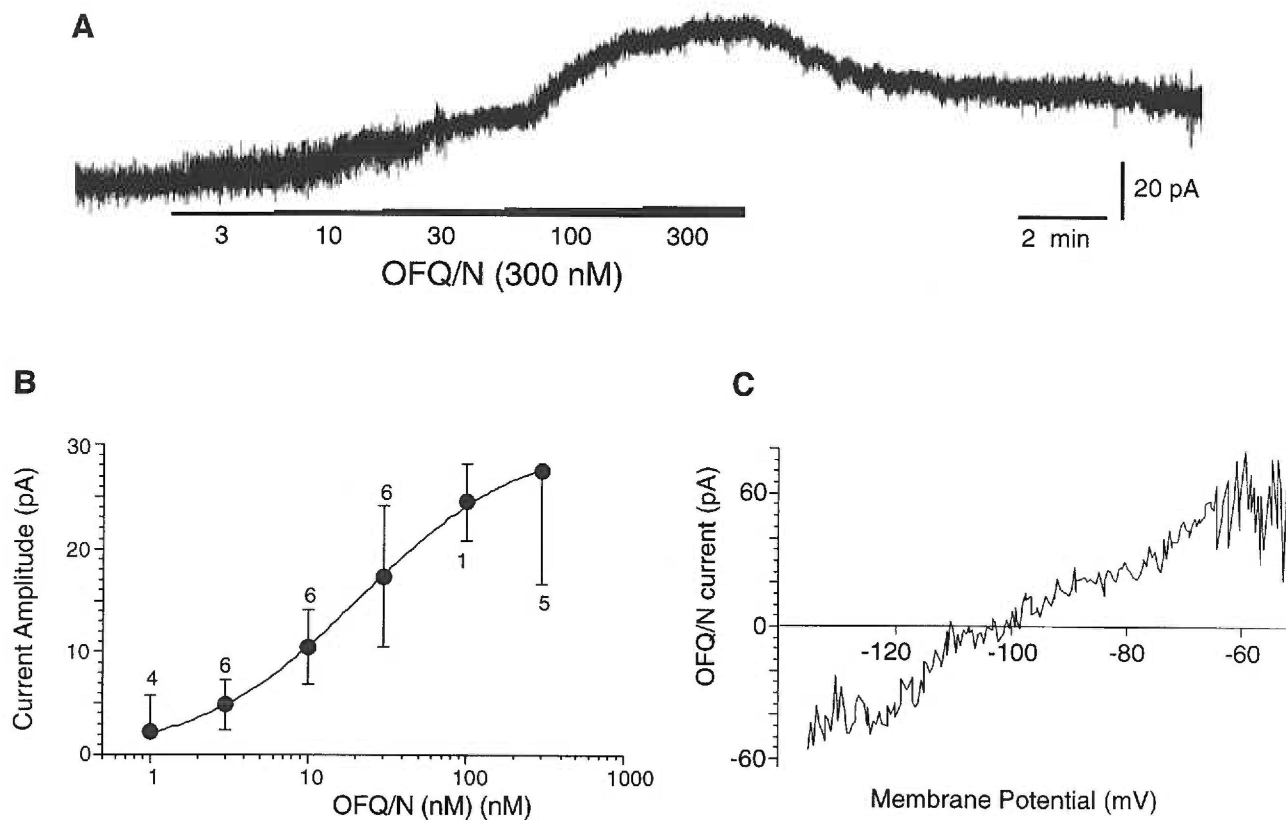


Figure 2. OFQ/N-activated a current in SCN neurons. A. OFQ/N induced an outward current when applied to an SCN neuron voltage-clamped at -60 mV. Note that the amplitude of the current was larger with increasing concentrations of OFQ/N (3 - 300 nM)_. B, Concentration-effect curve for OFQ/N-activated currents is shown. The EC_{50} was estimated to be 22.3 nM. The numerals above and below the SEM bars indicate the number of cells recorded at each OFQ/N concentration. C, Reversal potential of OFQ/N-induced current.

Figure 3

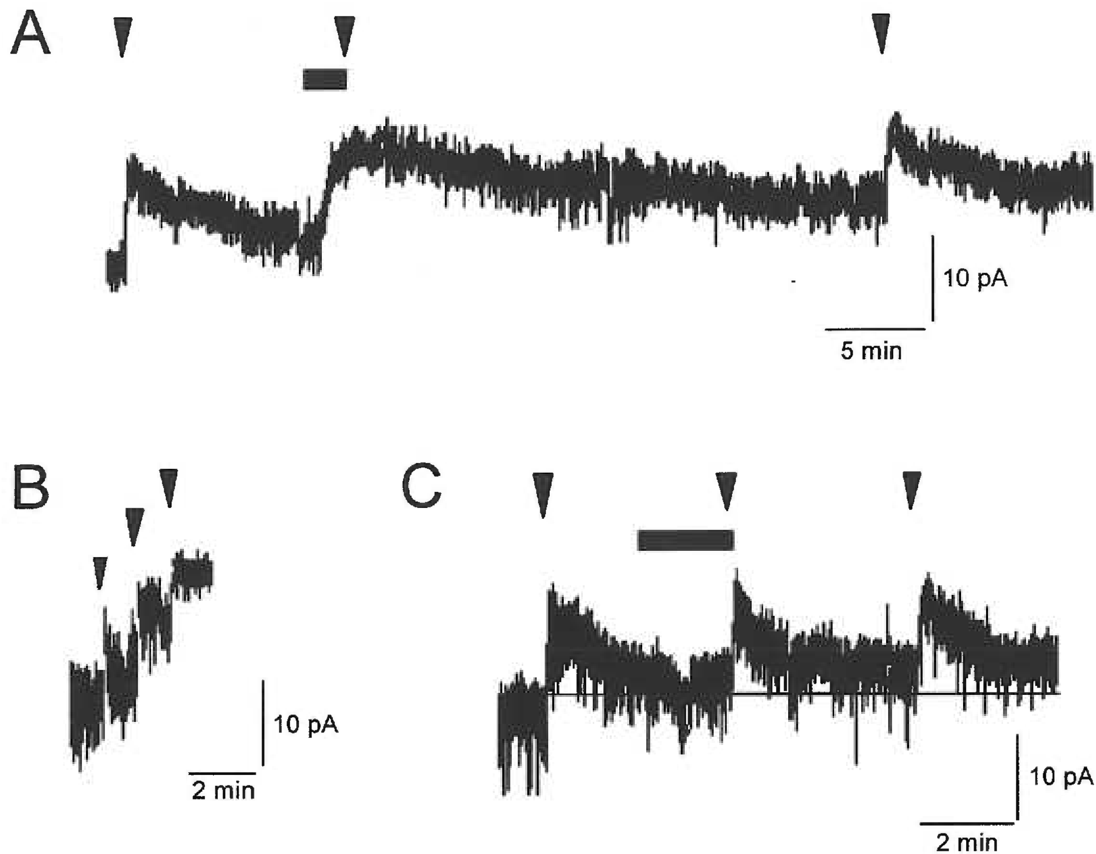


Figure 3. Effects of [Phe$^1\psi$(CH_2-NH)Gly^2]OFQ/N(1-13)NH_2 and naloxone on OFQ/N-activated currents. A, Currents activated by a 2 sec application of OFQ/N (300 nM; arrowheads) are shown. [Phe$^1\psi$(CH_2-NH)Gly^2]OFQ/N(1-13)NH_2 (3 μM; horizontal bar) was bath applied. Note that the OFQ/N antagonist activated an outward current and the subsequent application of OFQ/N did not activate an additional current. These data suggest that the OFQ/N antagonist has both agonist and antagonist activity at NOR. B, Multiple application of OFQ/N (300 nM, 2 sec; arrowheads) continued to activated additional current. This demonstrates that the OFQ/N (300 nM, 2 sec) application did not saturate the potential OFQ/N-activated currents. Therefore, the block of the OFQ/N-activated current by [Phe$^1\psi$(CH_2-NH)Gly^2]OFQ/N(1-13)NH_2 _was not attributable to a saturation of NOR stimulation. C, Naloxone (1 μM; horizontal bar) did not alter the amplitude of OFQ/N-activated currents (300 nM; arrowheads).

activates a K^+ current in SCN neurons via a unique receptor that is not a member of the naloxone-sensitive opioid receptor family. In addition, [Phe$^1\psi$(CH_2-NH)Gly^2]OFQ/N(1-13)NH_2 is a partial agonist of NOR in the SCN with actions similar to those observed in the spinal cord (Xu *et al.*, 1998).

Effect of OFQ/N on Circadian Phase

Because both OFQ/N immunoreactivity and its receptor mRNA were detected in the SCN and OFQ/N activated a robust K^+ current in the slice preparation, we sought to determine whether OFQ/N was capable of altering the phase of the circadian clock in vivo. OFQ/N (50 pmol) or vehicle was injected into the SCN region of free-running Syrian hamsters maintained in constant darkness. Groups of hamsters received injections at four different circadian times: CT2 (10 hours prior to anticipated activity onset), CT8 (4 hours prior to anticipated activity onset), CT14 (2 hours after anticipated activity onset) and CT20 (6 hours after anticipated activity onset). In all cases, OFQ/N injection failed to significantly alter circadian phase (phase shifts: CT2 = 5.7 ± 4.5 min, n = 6; CT8 = 6.0 ± 14.4 min, n = 4; CT14 = 8.0 ± 5.1 min, n = 6; CT20 = 2 ± 10.9 min, n = 6). These effects of OFQ/N were not significantly different from the effects of vehicle administered at the same four circadian times (ANOVA, $p>0.05$).

In rodents maintained under constant darkness, a single, brief exposure to light during the latter half of the subjective night results in a permanent phase advance of the circadian activity rhythm (DeCoursey, 1964; Rea *et al.*, 1993). Certain SCN neurotransmitters have been shown to modify this response to light, including serotonin and neuropeptide Y (Rea *et al.*, 1993, 1994; Weber *et al.*, 1995a,b). In the present study, light exposure after vehicle injection at CT19 resulted in a phase advance of 62 ± 5 min (Fig. 4). The injection of less than 5 pmol of OFQ/N prior to light exposure failed to alter the magnitude of the light-induced phase advance (0.1 pmol = 61.8 ± 6.8 min (n = 6); 0.5

Figure 4

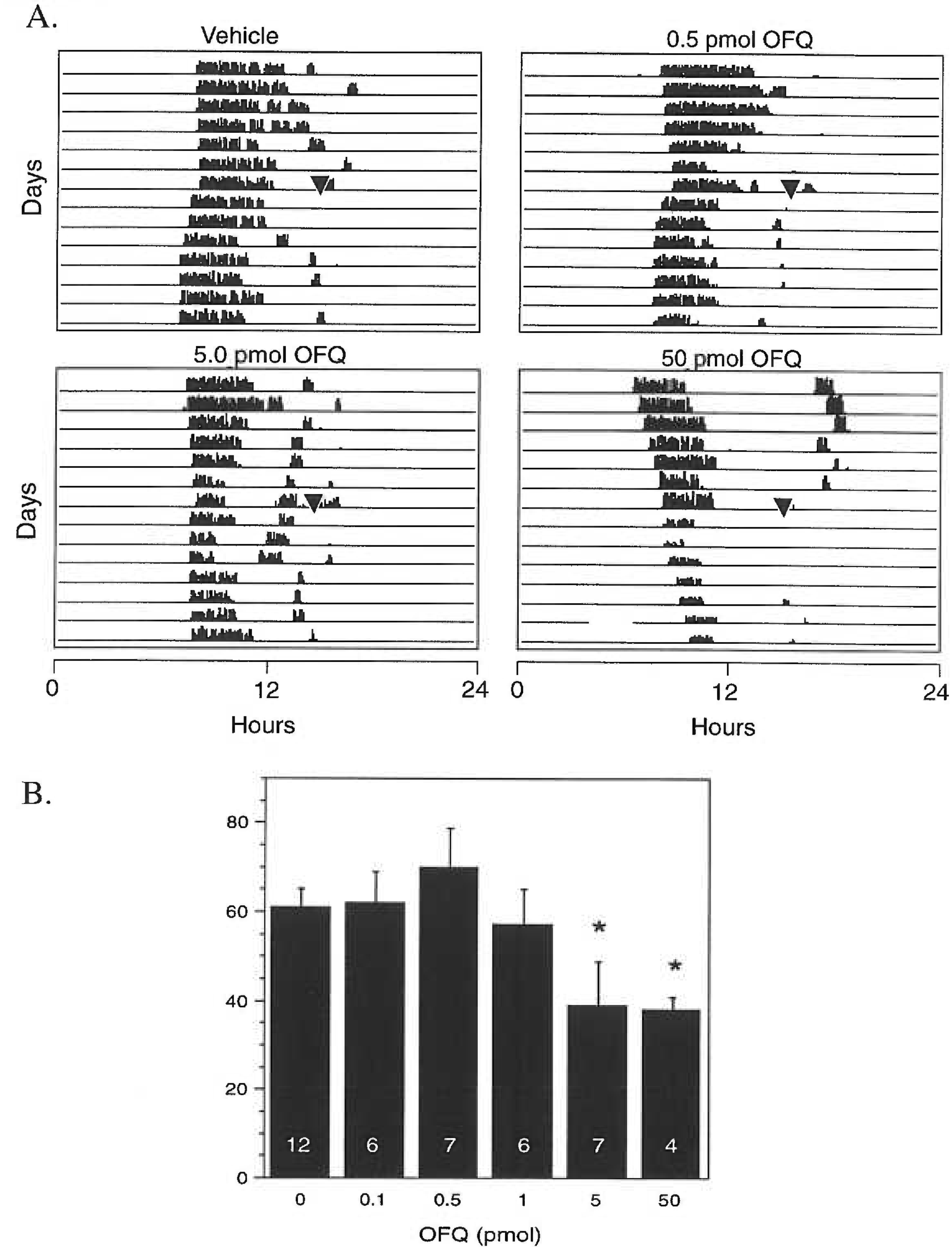


Figure 4. Effects of OFQ/N on light-induced phase shifts. A, Actographs showing that injection of OFQ/N 10 min before a 10 min light exposure at CT19 reduced the phase shift induced by the light pulses. B. Dose-dependent effect of local administration of OFQ/N on the light-induced phase shifts. Data represents the means ± S.D. and of the number of determinations are indicated on each bar.

pmol = 70.3 ± 8.3 min (n = 7); 1.0 pmol = 57.0 ± 7.3 min (n = 5); ANOVA, $p < 0.96$). However, injection of 5.0 pmol of OFQ/N resulted in a 39% reduction in the magnitude of the light-induced phase advance [39 ± 10 min, n = 7; $p < 0.05$]. A similar reduction in the phase-advance was obtained with injection of 50 pmol of OFQ/N [38 ± 3 min, n = 4; $p < 0.05$].

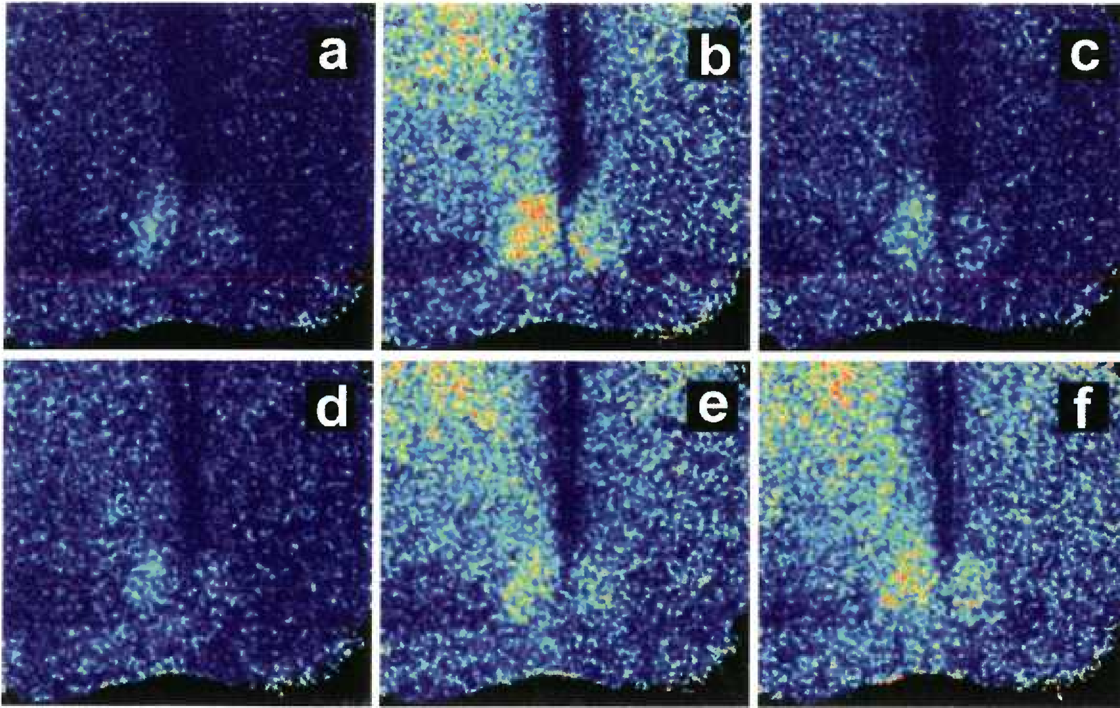
Inhibition of NMDA receptor-mediated Ca^{2+} influx by OFQ/N

An important component of light-induced phase shifts of the circadian clock is the activation of NMDA receptors by glutamate released from the RHT and an increase of intracellular Ca^{2+} (Ding *et al.*, 1994; MacDermott *et al.*, 1986). We therefore tested whether OFQ/N could modify the intracellular levels of Ca^{2+} in SCN neurons. NMDA (100 μM) increased the intracellular Ca2+ levels in the SCN 16.3 ± 2.7% (mean ± S.E., n = 4) (Fig. 5). Application of OFQ/N (1 μM) alone produced a small decrease in intracellular Ca2+ (6.0 ± 1.5%; mean ± S.E., n = 4). However, following OFQ/N (1 μM), the NMDA-induced increased intracellular Ca^{2+} was reduced 40% (9.9 ± 2.7%, mean S.E.M., n = 4). The NMDA response slowly returned to NMDA alone levels. In contrast, OFQ/N did not alter the NMDA-induced increase in intracellular Ca^{2+} in the anterior hypothalamic area (NMDA - 16.1 ± 5.5% versus OFQ/N and NMDA - 13.9 ± 4.9%). OFQ/N may therefore act to reduce the effects of light on the circadian clock by inhibiting the NMDA receptor-induced increase of intracellular Ca^{2+}.

Discussion

The data presented in this report demonstrate that (1) the OFQ/N peptide and its receptor are present in the SCN, (2) OFQ/N alters the activity of the majority of SCN neurons, and (3) OFQ/N injected into the SCN region modulates the response of the

Figure 5



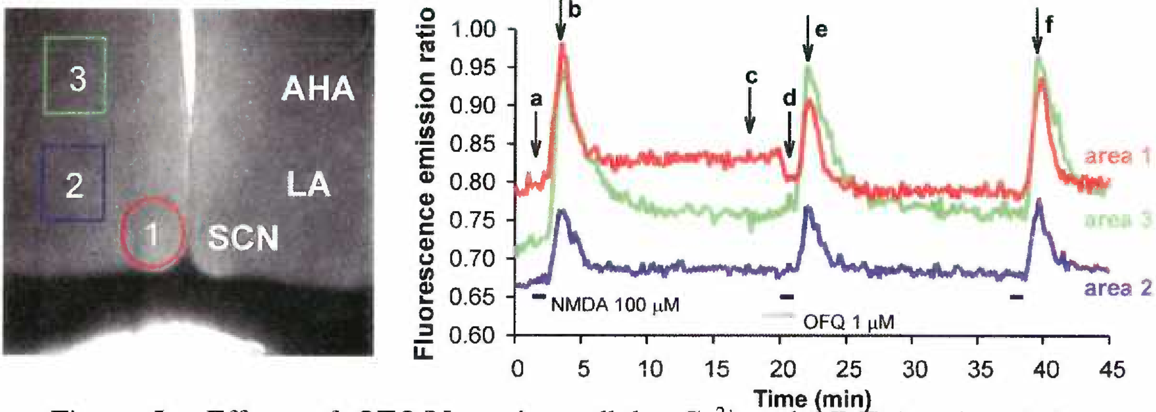


Figure 5. Effects of OFQ/N on intracellular Ca^{2+} and NMDA-activated increases in intracellular Ca^{2+}. A, Pseudocolor ratio images of NMDA-evoked increases in intracellular Ca^{2+} concentration. The individual frames are a) control, b) NMDA (100 μM), c) washout of NMDA, d) application of OFQ/N (1 μM), e) NMDA (100 μM), and f) control NMDA response. B. Normal illumination of an SCN slice shown with the regions of analysis outlined. The graph represents a continuous plot of the data the images shown in A correspond to the letters. The images are taken once each 10 sec. The individual images in A correspond to the labels in B. Abbreviations AHA anterior hypothalamus, LA lateral-anterior hypothalamus, SCN suprachiasmatic nucleus.

circadian clock to photic stimuli (Figs. 1,2,4). These observations argue strongly for a role of OFQ/N as a modulatory neuropeptide in the SCN. Furthermore, since OFQ/N is synthesized as part of a precursor protein that is not found in the SCN, the ppOFQ/N must be synthesized by neurons in brain regions that project to and innervate the SCN. Additional research will be required to understand the physiological functions of OFQ/N in the SCN and describe the OFQ/N projection pathways.

OFQ/N inhibited the light-induced phase shifts of the onset of wheel running activity of Syrian hamsters (Fig. 4). In contrast, OFQ/N did not directly phase shift the circadian clock. These data suggest that the signal transduction pathways, which are coupled to NOR, are not directly capable of modifying the timing of the circadian clock. Nocturnal light phase advances the circadian clock when applied during the latter portion of the subjective night (DeCoursey, 1964; Rea *et al.*, 1993, 1994; Weber *et al.*, 1995a,b). This phase advance is mediated by a rise of intracellular Ca^{2+} through activation of NMDA receptors by glutamate released from the RHT (Ding *et al.*, 1994; MacDermott *et al.*, 1986). Elevated intracellular Ca^{2+} activates nitric oxide synthase, resulting in increased nitric oxide production (Ding *et al.*, 1994). Nitric oxide synthesis and release are required for both light- and glutamate- induced phase shifts to occur (Ding *et al.*, 1994; Weber *et al.*, 1995a). Light-induced phase advances also require cyclic GMP-dependent protein kinase G activity, which is presumed to be increased as a consequence of the elevation of cyclic GMP levels by nitric oxide (Ding *et al.*, 1994,1998; Weber *et al.*, 1995a,b). Thus, inhibition of the increase of intracellular Ca^{2+} should attenuate the light induced phase advance. In the present report, we show that, in addition to attenuating photic phase advances, OFQ/N dose-dependently inhibits the NMDA receptor mediated increase in intracellular Ca^{2+} on SCN neurons by a mechanism that remains to be described (Fig. 5). Therefore, we propose that OFQ/N acts as a negative modulator of RHT neurotransmission through inhibition of the rise of intracellular Ca^{2+} mediated by NMDA receptors on retinorecipient SCN neurons, resulting in an attenuation of photic phase adjustments of the circadian clock.

OFQ/N produced a maximum 39% reduction in the magnitude of the light-induced phase advance (Fig. 3). This effect of OFQ/N is in contrast with excitatory amino acid antagonists that have the ability, at proper concentrations, to completely block the light-induced phase shift (Rea *et al.*, 1993). OFQ/N's inability to completely block the light-induced phase shift may be due in part to the fact that OFQ/N was only injected into one side of the SCN. Bilateral activation of NORs in the SCN may be required for the complete block of the light-induced phase shift. A second possibility is that the mechanism of OFQ/N action is different from other neuromodulators and OFQ/N does not have the ability to produce a complete block of the light induced phase shifts. This is consistent with the observation that OFQ/N only attenuated the NMDA-induced rise in intracellular Ca^{2+} 40% (Fig. 5).

SCN neurons are heterogeneous in their morphology, afferent synapses and their responses to neurotransmitters. The percentage of SCN neurons that responded to OFQ/N (88%) was higher than has been observed for other neurotransmitters in the SCN. In our experience 39% of SCN neurons respond to melatonin, 28% to serotonin, and 35% to baclofen; percentages that are similar to those reported by others (Jiang *et al.*, 1995a,b). For example, baclofen inhibited the single unit discharges of 65% of SCN neurons sampled while producing an outward current in only 35% of neurons (Liou *et al.*, 1990; Jiang *et al.*, 1995a). Melatonin application generated an outward current in only 35% of cells and inhibited the firing of 39-100% of SCN neurons depending on the preparation and the time of day (Stehle *et al.*, 1989; Mason and Brooks, 1988; Shibata *et al.*, 1989). 5-HT application activated an outward current in only 27% of cells and inhibited firing in only 49-56% of cells (Mason and Brooks, 1988; Meijer and Groos, 1988; Miller and Fuller, 1990). The fact that 88% of SCN neurons respond to OFQ/N make it one of the most ubiquitous modulators of the activity of SCN neurons.

NOR mRNA is expressed and the OFQ/N peptide dose-dependently activates outward currents in SCN neurons (Fig. 1,2,3). OFQ/N modulates the membrane

conductance and activity of SCN neurons by activating a K^+ current which would hyperpolarize the membrane potential making the SCN neurons less excitable (Fig. 2). NORs are coupled to G_i type G-proteins and inhibit forskolin-stimulated adenylyl cyclases and voltage-gated Ca^{2+} channels via a pertussis toxin-sensitive mechanism (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995). Further work will be needed to determine if NORs on SCN neurons are coupled to similar effector systems. When expressed in Xenopus oocytes NORs can couple to G-protein-activated K^+ channels consisting of Kir3.1 and Kir3.4 subunits (Matthes *et al.*, 1996). Kir3 channels are G-protein-activated K^+ channels that are directly stimulated by $\beta\gamma$ G-protein subunits giving inwardly rectifying K^+ currents (Kofuji *et al.*, 1995). OFQ/N also has been reported to activate an inwardly rectifying K^+ current in dorsal root ganglion, periaquaductal gray and arcuate neurons (Vaughan and Christie, 1996; Vaughn *et al.*, 1997; Wagner *et al.*, 1998). It is likely that the K^+ channels that are known to be present on SCN neurons (Karschin *et al.*, 1994; Dißmann *et al.*, 1996).

The OFQ/N peptide is expressed in the SCN, whereas ppOFQ/N mRNA is not, suggesting that ppOFQ/N is synthesized by neurons in brain regions that project to the SCN (Fig. 1B-D). The identity of the afferent projection pathway or pathways remains unknown. However, several brain regions are implicated as potential sources of the OFQ/N projection by virtue of their neuroanatomical and functional relationships with the SCN. Entrainment of the circadian pacemaker is tightly regulated by environmental lighting cues that are conveyed to the SCN by both direct and indirect pathways. The intergeniculate leaflet (IGL) is a small region located between the dorsolateral geniculate and the ventrolateral geniculate that receives dense input from the retina and projects via the geniculohypothalamic tract to the SCN (Card and Moore, 1989). Lesioning the IGL reduces the rate of re-entrainment following phase advances or phase delays (Harrington and Rusak, 1986; Pickard *et al.*, 1987). Four types of neurons exist within the IGL and the two best-studied neurotransmitters found in some of these neurons are GABA and neuropeptide Y (Shinohara *et al.*, 1993; Morin and Blanchard, 1995). There are also IGL

neurons that express an additional peptidergic neuromodulator which remains to be identified (Card and Moore, 1989). The presence of ppOFQ/N mRNA in the VGL suggests that OFQ/N neurons may exist in the IGL. However, the resolution of the *in situ* hybridization studies was not sufficient to positively identify the IGL. The lateral septum, preoptic area and median raphe are regions which also have dense staining for ppOFQ/N mRNA and provide afferent projections to the SCN (Moga and Moore, 1997; Darland *et al.*, 1998). Future experiments will be performed which will lesion these regions to determine if OFQ/N levels in the SCN are decreased.

OFQ/N was originally identified as the endogenous ligand for a receptor with homology to the classical opioid receptors. Due to this apparent evolutionary relatedness most studies to date have focused on a role for the peptide and its receptor in nociception and feeding behavior (Mogil *et al.*, 1996a; Pomonis *et al.*, 1996). The present data demonstrate that OFQ/N can modulate the activity of SCN neurons and that OFQ/N has important actions in brain regions other than those involved in nociception. NORs are located on neurons of the SCN and their activation increases the membrane conductance by activation of a K^+ current (Fig. 2). In addition, injection of OFQ/N into the SCN region inhibits photic phase advances of the circadian activity rhythm. These observations suggest that OFQ/N alters the activity of retinorecipient SCN neurons by activation of a K^+ current, resulting in hyperpolarization of the membrane potential and inhibition of the response of the postsynaptic neuron to light-activated RHT neurotransmission. In addition, OFQ/N may act as a negative modulator of RHT neurotransmission through inhibition of the rise of intracellular Ca^{2+} mediated by NMDA receptors on retinorecipient SCN neurons, resulting in an attenuation of photic phase adjustments of the circadian clock. In conclusion, the heptadecapeptide OFQ/N has direct actions on SCN neurons, and may serve as a modulator of the phase-regulatory effects of light on the circadian clock.

Acknowledgments

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Chapter 3

Regulation of Nociceptin (N)/Orphanin FQ (OFQ) Peptide Levels in the Mouse Hypothalamus by Restraint Stress, Corticotropin-Releasing Hormone and Glucocorticoids

Regulation of Nociceptin (N)/Orphanin FQ (OFQ) Peptide Levels in the Mouse Hypothalamus by Restraint Stress, Corticotropin-Releasing Hormone and Glucocorticoids

Denise I. Quigley[a], David K. Grandy[b], Malcolm J. Low[c], Mary Stenzel-Poore[d] and Richard G. Allen[e]

[a]Department of Molecular and Medical Genetics, [b]Department of Physiology and Pharmacology, [c]Vollum Institute, [d]Department of Molecular Microbiology and Immunology, and [e]The Center for Research on Occupational and Environmental Toxicology, Oregon Health Sciences University, Portland, OR 97201

Correspondence to:
Richard G. Allen, Ph.D.
Center for Research on Occupational and Environmental Toxicology
Oregon Health Sciences University
3181 SW Sam Jackson Park Rd.
Portland, OR 97201
Phone: 503-494-2508
Fax: 503-494-6831
Allenr@ohsu.edu

Running title: HPA axis modulation of N/OFQ peptide

Abstract

Nociceptin/Orphanin FQ (N/OFQ) is a recently discovered neuropeptide that is structurally related to the opioid peptides, but does not bind to opioid receptors with high affinity. Recent studies have suggested that in addition to its proposed anti-opioid properties, N/OFQ can play a modulatory role in integrating responses to stressful stimuli; in particular, the peptide functions as an anxiolytic. We have studied effects of stressors and stress hormones on N/OFQ and β-endorphin peptide levels in the mouse hypothalamus using sensitive and specific radioimmunoassays. Hypothalamic extracts prepared from mice that received chronic injections of dexamethasone (DEX), a synthetic glucocorticoid, contained significantly less N/OFQ peptide than control animals. However, DEX treatment had no effect on hypothalamic β-endorphin peptide levels. Transgenic mice that overproduce corticotrophin-releasing hormone (CRH) exhibit elevated plasma corticosterone levels. A small apparent increase in N/OFQ peptide levels was detected in hypothalamic extracts from CRH-overproducing mice compared to control animals. Additionally, β-endorphin peptide levels in hypothalamic extracts from CRH-overproducing mice were not significantly different compared to the controls. When mice were subjected to chronic restraint stress they exhibited elevated plasma corticosterone and reduced hypothalamic N/OFQ levels compared to control animals. In contrast, hypothalamic β-endorphin levels were not altered by this chronic restraint stress paradigm. To further elucidate the circuitry by which N/OFQ peptide levels are regulated by the HPA axis we determined hypothalamic N/OFQ peptide levels in mutant mice that lack β-endorphin. No significant difference in hypothalamic N/OFQ peptide levels was detected in these animals compared to control animals. Collectively, these results suggest that stress, glucocorticoids and CRH, but not β-endorphin, modulate N/OFQ peptide levels in the mouse hypothalamus.

Key Words Orphanin FQ/Nociceptin, Stress, Corticotropin-releasing hormone, glucocorticoids, HPA axis, Radioimmunoassay

Introduction

In addition to the three classical opioid receptor subtypes, μ, δ and κ, an orphan "opioid-like" G-protein coupled receptor has been identified (Bunzow *et al.*, 1994; Mollereau *et al.*, 1994; Nishi *et al.*, 1994; Henderson and McKnight, 1998; Darland *et al.*, 1998; Harrison and Grandy, 2000). Despite significant sequence similarity this receptor fails to bind any of the opioid peptides with high affinity (Bunzow *et al.*, 1994). nociceptin (N), also called Orphanin FQ (OFQ), a seventeen amino acid peptide bearing sequence homology to dynorphin A, has been identified as the endogenous ligand for this receptor, now referred to as the nociceptin/orphanin FQ receptor (NOR) (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995). Multiple cellular responses are evoked by N/OFQ binding to the NOR, including inhibition of adenylyl cyclase, activation of inwardly rectifying K^+ channels and inhibition of voltage-sensitive Ca^{2+} channels (Henderson and McKnight, 1998; Darland *et al.*, 1998; Harrison and Grandy, 2000; Meunier *et al.*, 1995; Reinscheid *et al.*, 1995).

Unlike the classical opioid peptides, N/OFQ administered supra-spinally does not appear to induce analgesia (Meunier *et al.*, 1995; Mogil *et al.*, 1999). Although N/OFQ has been implicated in the production of hyperalgesia when injected intracerebroventricularly (i.c.v.) in mice, N/OFQ possesses anti-opioid properties including the ability to functionally reverse stress-induced analgesia (SIA) (Mogil *et al.*, 1996b; Grisel *et al.*, 1996). Depending on the dose, N/OFQ administered i.c.v. has been shown to impair (Devine *et al.*, 1996) or increase locomotion (Florin *et al.*, 1996). N/OFQ has also been implicated in reward behavior pathway due to high receptor expression in the nucleus accumbens and its ability to decrease dopamine release in the accumbens following i.c.v. administration in anesthetized rats (Murphy *et al.*, 1996). Furthermore, N/OFQ administered i.c.v. has been shown to have anxiolytic properties and to attenuate behavioral responses to stress (Jenck *et al.*, 1997; Griebel *et al.*, 1999). Additionally, increased anxiety-like behavior and impaired adaptation to stressful stimuli is seen in N/OFQ-deficient mice (Köster *et al.*, 1999). While

N/OFQ appears to influence a number of physiological pathways, a single functional role of N/OFQ remains to be elucidated (for reviews see Henderson and McKnight, 1998; Darland *et al.*, 1998; Harrison and Grandy, 2000; Walker and Koob, 1997; Taylor and Dickenson, 1998).

N/OFQ immunoreactivity is abundant in the hypothalamus, a key component of the stress axis (Darland *et al.*, 1998; Quigley *et al.*, 1998). Glucocorticoid receptor (GR) immunoreactivity co-localizes with ppN/OFQ mRNA in regions of the hypothalamus, including the paraventricular nucleus, arcuate nucleus, dorsomedial nucleus and the lateral hypothalamic area (Darland *et al.*, 1998; Morimoto *et al.*, 1996). The relative abundance of N/OFQ in the hypothalamus and co-localization with GR suggests that ppN/OFQ mRNA expression may be modulated in response to stress. Based on the observations that N/OFQ has anxiolytic properties and reverses opioid-mediated stress induced analgesia (SIA) we hypothesized that hypothalamic N/OFQ peptide levels may be regulated either by activation or alteration of the hypothalamic-pituitary-adrenal (HPA) axis.

In the present studies we utilized several stress-related paradigms to examine their effects on hypothalamic N/OFQ and β-endorphin peptide levels. We found that chronic treatment with the synthetic glucocorticoid dexamethasone (DEX) resulted in reduced hypothalamic N/OFQ peptide levels, but had no effect on β-endorphin peptide levels. In transgenic mice that overproduce corticotrophin-releasing hormone (CRH), a principal regulator of the stress response, we detected an increase in N/OFQ hypothalamic peptide levels. These animals exhibit endocrine abnormalities involving the HPA axis, including increased plasma ACTH and glucocorticoids (Stenzel-Poore *et al.*, 1996). Mutant mice that lack β-endorphin, but retain all other proopiomelanocortin (POMC)-derived peptides (Rubinstein *et al.*, 1996), showed no difference in their hypothalamic content of N/OFQ peptide compared to that of wild type animals. Finally, chronic restraint stress resulted in a reduction in hypothalamic N/OFQ peptide immunoreactivity but had no effect on hypothalamic β-endorphin peptide levels. Taken together, these findings suggest that

hypothalamic N/OFQ peptide levels can be modulated by specific perturbations of the HPA-axis.

Materials and Methods

Animals

Wild type male and female C57BL/6 mice obtained from Jackson Laboratories were used in the restraint stress and dexamethasone treatment experiments described below. All animals were given food and water ad libitum and maintained on a 12 hour light/dark cycle. Experiments involving animals were performed in accordance with the NIH guidelines for the Care and Use of Laboratory Animals.

CRH transgenic animals: Male transgenic mice back-crossed to the C57BL/6 background for 8 generations were genotyped using PCR with transgene-specific primers to amplify tail genomic DNA (Stenzel-Poore *et al.*, 1996). Transgenic mice as well as age and sex matched controls were housed individually in covered cages the night before sacrifice and taken individually to a separate room for sacrifice to control for environmental stressors. Trunk blood and hypothalamic tissues were collected to measure serum corticosterone, N/OFQ and β-endorphin peptide levels.

Mutant Mice Lacking β-endorphin: Male and female homozygous mutant mice, backcrossed to the C57BL/6 genetic background for nine generations and matched wild type control animals were sacrificed and hypothalamic tissues collected to measure N/OFQ peptide levels (Rubinstein 1996). Mutant mice express a truncated proopiomelanocortin (POMC), which lacks β-endorphin, but retains the rest of POMC. Hypothalamic β-endorphin peptide levels were also measured to confirm the mutant genotype.

Dexamethasone Treatment

Wild-type male C57BL/6 mice at 7-8 weeks of age were injected subcutaneously with 1 mg/kg dexamethasone (DEX) (Sigma) twice daily, for 8 days. Control animals were injected subcutaneously with saline twice daily or were left untreated for 8 days. On the eighth day the animals were housed individually, in covered cages, overnight to minimize stress before sacrifice. On the ninth day, animals were sacrificed two hours after receiving the final DEX or saline injection. Animals were taken individually to a separate room for sacrifice. Trunk blood and hypothalamic tissues were collected to measure serum corticosterone, N/OFQ and β-endorphin peptide levels.

Restraint Stress

Age-matched wild-type female C57BL/6 mice were restrained in clear plastic cylinders for one hour a day for eight consecutive days. The mice were not completely immobilized in the restrainers, but were unable to turn around 180°. Control animals were untreated for the duration of the study. Animals were sacrificed on the eighth day, two hours after the cessation of the last restraint period. Animals were not housed individually and no special precautions were taken to minimize stress during sacrifice. Trunk blood and hypothalamic tissues were collected to measure serum corticosterone, N/OFQ and β-endorphin peptide levels.

Radioimmunoassay (RIA) of OFQ/N, β-endorphin and corticosterone:

All basic β-endorphin RIA procedures were carried out as previously described (Allen *et al.*, 1978). The N/OFQ RIA was performed as described (Quigley *et al.*, 1998). Synthetic peptides were obtained from Peninsula Laboratories and Phoenix Pharmaceuticals. Corticosterone (CORT) levels were measured by RIA using a rat/mouse corticosterone RIA kit (ICN Biochemicals). The detection limits for the RIAs are 4-6

pg/tube for the N/OFQ and β-endorphin assays and 5-10 ng/ml for the corticosterone assay.

Tissue Processing

Individual hypothalamic blocks (10-15 mg) bordered rostrally by the optic chiasm, caudally by the mammillary body, laterally by the optic tract and dorsally by the top of the third ventricle were weighed and extracted in 10% acetic acid containing 3 mM phenylmethylsulfonylfluoride (PMSF) and 0.5 mg/ml Bovine Serum Albumin (Sigma). Extracts were frozen and thawed 3 times on dry ice and centrifuged for five minutes at 10,000 RPM in a table-top centrifuge. The supernatants were lyophilized and stored until subjected to RIA. Serum obtained from core blood was stored at -20°C until assayed.

Statistics

Statistical analyses were determined using Statview™ software.

Results

To determine whether high circulating levels of glucocorticoids alter hypothalamic N/OFQ peptide levels, C57BL/6 male mice were treated for 8 days with DEX as described in the methods. Following treatment hypothalamic N/OFQ, β-endorphin peptide levels and plasma CORT levels were measured by RIA. Figure 1A shows that baseline plasma CORT levels in saline-injected and non-injected control animals ranged from 5-10 ng/ml while plasma CORT was undetectable in the DEX-treated animals. Hypothalamic N/OFQ peptide levels were significantly reduced in DEX-treated animals compared to non-injected control animals (Fig. 1B). The reduction in hypothalamic N/OFQ peptide levels was also significant in the DEX treated animals compared to that of the saline-injected control

animals. In contrast, hypothalamic β-endorphin peptides levels were not significantly different in DEX-injected, saline-injected or non-injected animals (data not shown). These results suggested that high circulating levels of glucocorticoids affect the content of N/OFQ but not β-endorphin peptide in the hypothalamus.

Increased production of CRH is a critical component of the response to stress and to examine the possible role of CRH in modulating hypothalamic levels of β-endorphin and N/OFQ, we used a transgenic mouse model in which CRH is overproduced. Plasma CORT levels in these animals are significantly elevated (approximately 140 ng/ml) compared to control animals (approximately 20 ng/ml) (Fig. 2A). Figure 2B shows that these animals have significantly elevated hypothalamic N/OFQ while β-endorphin peptide levels remained unchanged (data not shown), suggesting that chronic over-production of CRH alters hypothalamic N/OFQ levels but has no effect on hypothalamic β-endorphin.

We hypothesized that hypothalamic N/OFQ peptide levels may fluctuate in the presence of environmental stressors and with the duration of the stressful stimuli. Plasma CORT was elevated in the chronically stressed treatment group compared to that of the matched controls (Fig. 3A). Additionally, hypothalamic N/OFQ peptide levels were reduced in the chronically stressed animals compared to controls (Fig. 3B), while hypothalamic β-endorphin peptide levels were not significantly different between the two treatment groups (data not shown). These results suggest that chronic stress has an effect on hypothalamic N/OFQ but not on hypothalamic β-endorphin peptide levels. To explore whether N/OFQ is regulated by β-endorphin we utilized mutant mice that express a truncated POMC precursor that lacks this neuropeptide. Figure 4 shows that hypothalamic N/OFQ peptide content in wild-type and homozygous mutant mice is not significantly different. Previous studies revealed no differences in serum corticosterone between wild-type and β-endorphin deficient mice (Rubinstein *et al.*, 1996). These results suggest that hypothalamic N/OFQ peptide levels and serum corticosterone are not altered by lack of β-endorphin.

Figure 1

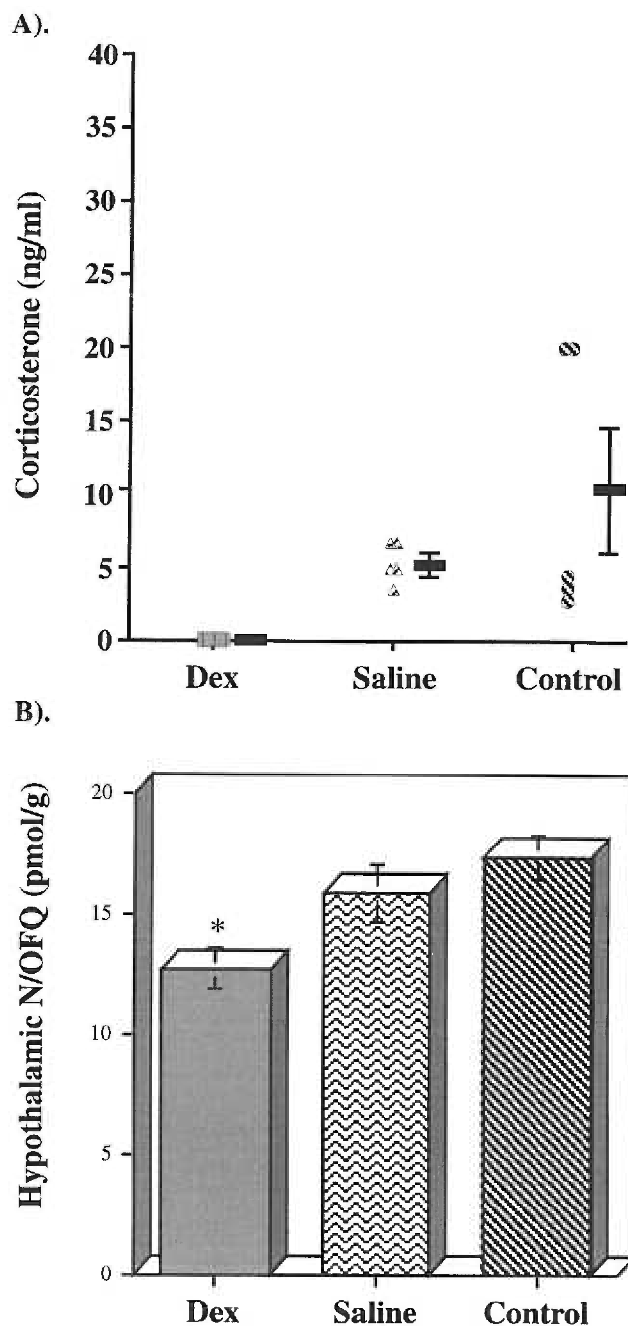

Figure 1. A) Corticosterone levels in DEX treated animals. Comparison of basal serum corticosterone levels in DEX treated, saline control and untreated control mice (n=5 each group). Serum samples were taken from unstressed mice and represent single serum corticosterone determinations. Error bars represent +/- S.E.M. Statistical analyses determined by ANOVA were p=0.002 dex compared to saline, p=0.06 dex compared to control and p=0.201 saline compared to control. B) Hypothalamic N/OFQ peptide levels following chronic DEX treatment. Values are expressed in pmol N/OFQ/g wet tissue weight +/- S.E.M. Statistical significance as determined by ANOVA is p=0.05 DEX compared to saline and p=0.007 DEX compared to control.

Figure 2

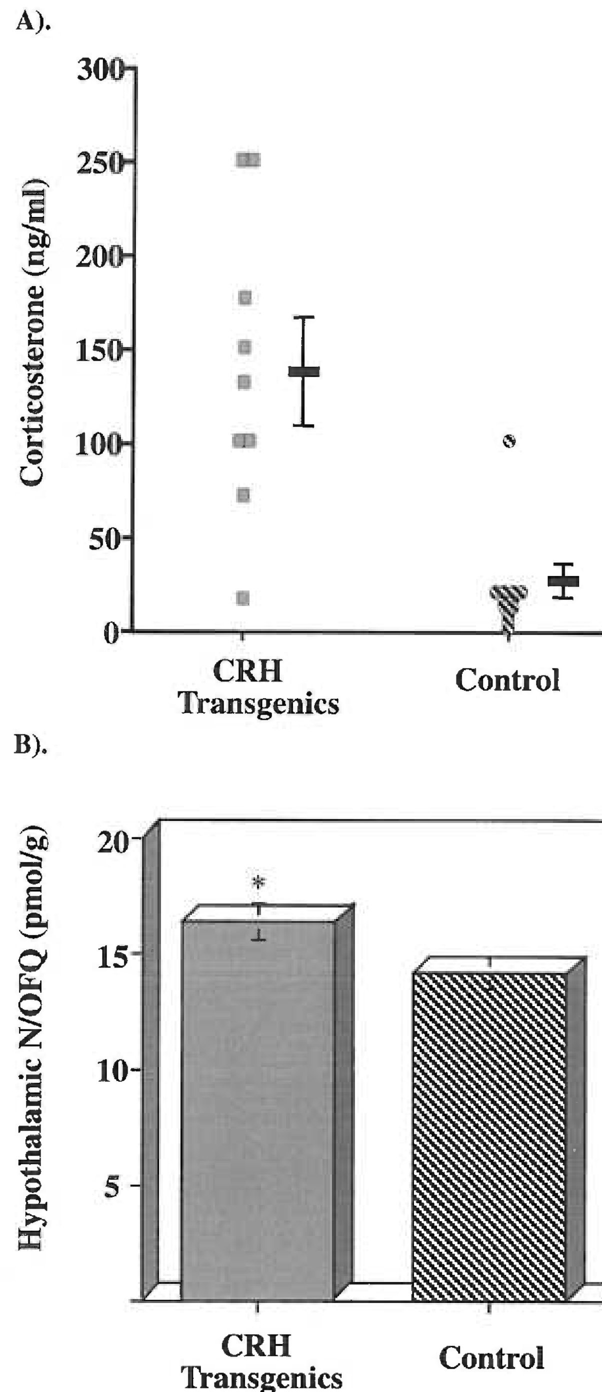

Figure 2. A) Corticosterone levels in transgenic animals that over exposes CRH. Comparison of basal serum corticosterone levels in CRH over expressing transgenic animals (n=9) and wild type controls (n=15). Plasma samples were taken from unstressed mice and represent single plasma corticosterone determinations. The error bars represent +/- S.E.M.; p=0.004. B) Hypothalamic levels of N/OFQ peptide in transgenic mice that overproduce CRH. Values are expressed in pmol peptide/g wet tissue weight +/- S.E.M. Statistical significance was p=0.01.

Figure 3

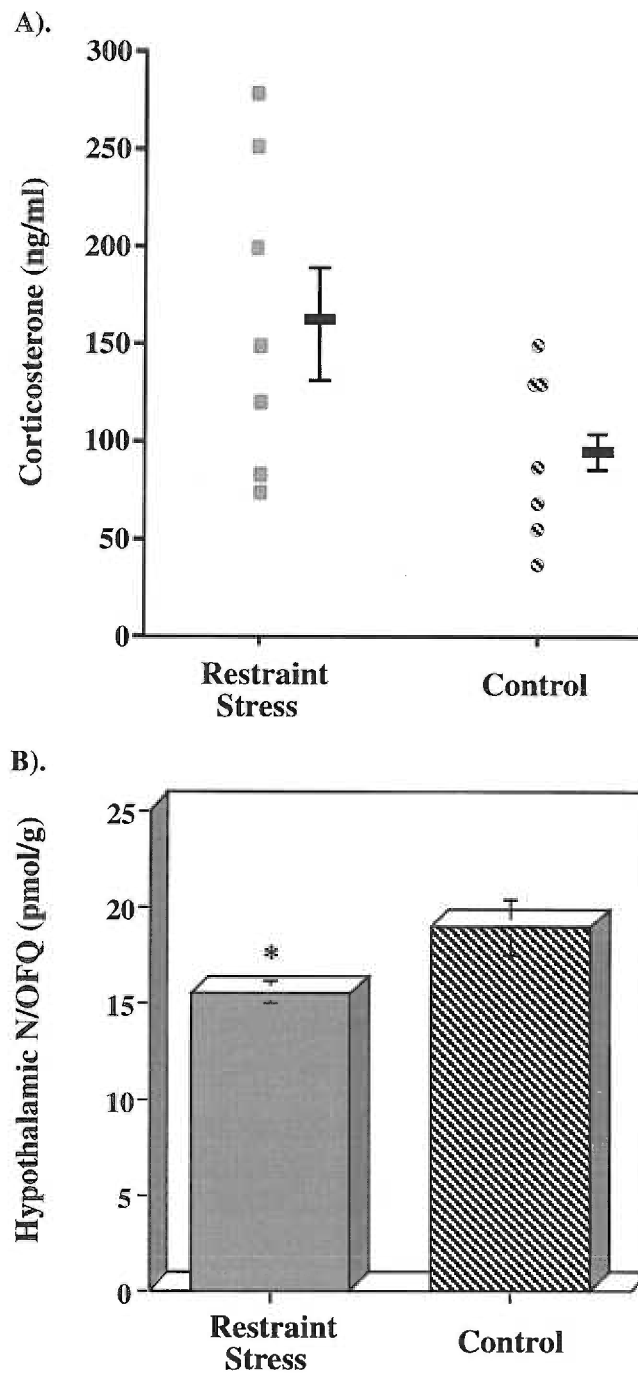

Figure 3. A) Serum corticosterone levels in mice subjected to chronic restraint stress. Plasma samples represent single serum corticosterone determinations measured in restraint stressed and control mice (n=7 each group). Statistical analysis determined by the Student's t-test was p=0.07. B) Hypothalamic peptide levels of N/OFQ in mice subjected to restraint stress. Values are expressed in pmol N/OFQ/g wet tissue weight +/- S.E.M. Statistical significance was p=0.01.

Discussion

The present studies, summarized in table 1, were designed to test specific effects of HPA axis activity on the regulation of hypothalamic N/OFQ peptide content. The HPA axis is activated in response to stressful stimuli, linking central nervous system signals to peripheral target gland activity. CRH release from the hypothalamus stimulates pituitary synthesis and secretion of ACTH, resulting in synthesis and release of adrenocortical hormones including glucocorticoids. Circulating glucocorticoids in turn feed back at the level of the pituitary gland, the hypothalamus and extrahypothalamic sites where they can inhibit the activity of the HPA axis. Because the N/OFQ peptide is abundant in the hypothalamus (Rubinstein *et al.*, 1996), has anxiolytic properties (Jenck *et al.*, 1997), and is a putative member of the opioid family of peptides, a peptide family known to modulate HPA axis activity (Akil *et al.*, 1984), it seemed reasonable to postulate that hypothalamic N/OFQ peptide content may be modulated by activity within the HPA axis.

Our results indicate that DEX administration significantly reduced N/OFQ immunoreactivity and had no effect on β-endorphin immunoreactivity in the hypothalamus. The DEX treatment provides very high circulating levels of glucocorticoids that inhibit HPA axis activity. Virtually undetectable levels of plasma corticosterone in the DEX treated animals confirms inactivity of the HPA axis. The results of this experiment suggest that high plasma glucocorticoids due to DEX administration reduced hypothalamic N/OFQ peptide content.

Over-expression of CRH resulted in elevated hypothalamic N/OFQ and a non-significant increase in hypothalamic β-endorphin. This result may be due to direct regulation of N/OFQ release by CRH. Despite high plasma corticosterone levels, which would normally inhibit the HPA axis, the CRH over-producing transgenic mice exhibit chronic activation of the HPA axis resulting from unrestrained CRH expression by the CRH transgene. Elevated N/OFQ peptide in the hypothalamus of these transgenic animals

Figure 4

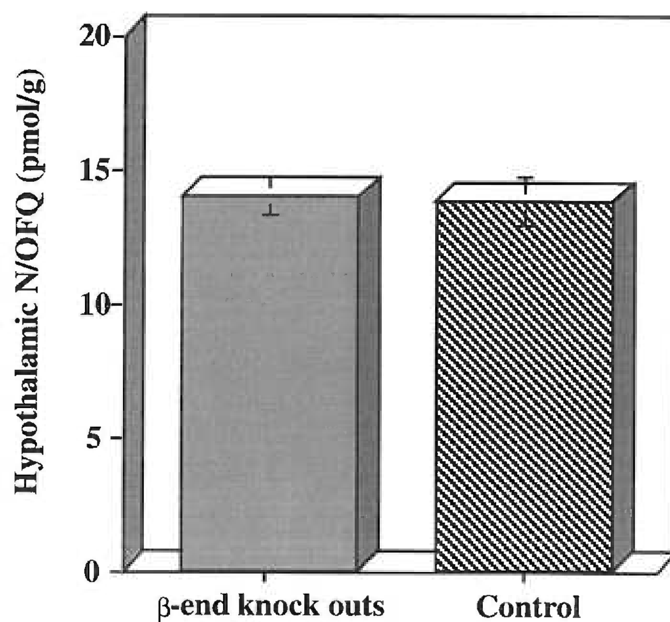

Figure 4. Hypothalamic N/OFQ peptide levels in mice lacking β-endorphin. N/OFQ peptide levels were measured by RIA in mutant (n=16) and wild type (n=15) mice. Values are expressed as pmol N/OFQ/g wet tissue weight +/- S.E.M.

Table 1

Treatment	**Peptide (change)**	
	N/OFQ	**β-endorphin**
+ DEX	↓	**NC**
+ CRH	↑	**NC**
- β-endorphin	**NC**	**absent**
Restraint stress	↓	**NC**

Table 1. A summary of the changes in hypothalamic N/OFQ and β-endorphin peptide content per treatment compared to control animals. ↑ = increase, ↓ = decrease, NC = no change.

may be behaviorally important because of the anxiolytic properties of N/OFQ (Jenck *et al.*, 1997). The elevation in N/OFQ peptide could counter balance the effects of elevated CRH, an anxiogenic peptide, and maintain homeostasis within the organism. Indeed, these CRH over producing mice demonstrate anxiogenic behavior (Stenzel-Poore *et al.*, 1994), thus a compensatory increase in anxiolytic neuropeptides might be expected in this model organism.

Chronic stress states result in opioid mediated inhibition of HPA axis activity and adaptation of the HPA axis such that stressful stimuli no longer produce opioid mediated analgesia (Akil *et al.*, 1984; Janssens *et al.*, 1995). Animals subjected to chronic stress in this experiment exhibited an apparent reduction in hypothalamic N/OFQ peptide compared to the control animals. This result is consistent with the idea that N/OFQ peptide levels are reduced with inhibition of HPA axis activity, as observed in the DEX experiment. Hypothalamic β-endorphin peptide levels are not significantly different between the animals subjected to chronic stress compared to controls, a result similar to that observed in the DEX experiment.

Mutant mice lacking β-endorphin showed no change in their hypothalamic N/OFQ immunoreactivy compared to control animals. These animals were utilized to determine whether N/OFQ levels may be altered in the absence of an endogenous opioid peptide. Although β-endorphin is derived from POMC, a key molecule mediating the stress response, these animals exhibit normal HPA axis activity and responses to restraint and ether stress (Rubinstein *et al.*, 1996). β-endorphin levels are not significantly affected by any of the stress paradigms we studied and the lack of β-endorphin does not appear to alter hypothalamic N/OFQ content. This result suggests that either these animals have adapted such that hypothalamic β-endorphin levels are unchanged or that hypothalamic N/OFQ and β-endorphin levels are not directly inter-dependent and are not similarly regulated by the stress response.

The results of the present studies may be summarized by a single unifying hypothesis: hypothalamic N/OFQ immunoreactivity is reduced in the presence of elevated glucocorticoids, except in the presence of excess CRH. From these experiments, one can not determine whether the alteration in hypothalamic N/OFQ peptide levels is due to biosynthesis or secretion. However, one possible explanation for the elevation or reduction in N/OFQ is that ppN/OFQ expression is modulated directly by glucocorticoid receptors (GR). Indeed, the promotor region of the ppN/OFQ gene contains potential binding sites for transcription factors including GR (Xie *et al.*, 1999). Hypothalamic regions assayed in our studies, including the paraventricular nucleus, arcuate nucleus, dorsomedial nucleus and the lateral hypothalamic area are rich in ppN/OFQ mRNA as well as GR (Darland *et al.*, 1998; Morimoto *et al.*, 1996). ppN/OFQ mRNA levels may be directly inhibited by GR resulting in a reduction of N/OFQ levels in these hypothalamic regions. This idea is consistent with the results of our DEX and restraint stress experiments in which circulating corticosterone levels are extremely high and hypothalamic N/OFQ immunoreactivity is reduced. The results obtained with the CRH over-producing animals and the acutely stressed animals suggest there is a threshold effect of this GR mediated inhibition of ppN/OFQ mRNA. That is, circulating corticosterone levels are elevated in these animals but not to the extent as seen in the DEX-treated and restraint stressed animals, therefore N/OFQ peptide levels are not reduced by GR mediated inhibition of ppN/OFQ message expression. This threshold effect might explain the reduction or elevation of hypothalamic N/OFQ by activation or inhibition of the HPA axis observed in these different experimental groups of animals.

Figure 5 represents a model for the circuitry suggested by the results of these experiments. CRH appears to stimulate an increase in hypothalamic N/OFQ, in contrast, glucocorticoids are a more potent negative regulator of hypothalamic N/OFQ content functioning downstream of the CRH influence. Similarly, glucocorticoids negatively regulate CRH secretion, as suggested by HPA axis circuitry. CRH and glucocorticoids

appear to have no effect on hypothalamic β-endorphin content, as hypothalamic β-endorphin peptide levels were unaffected in the paradigms tested here. Moreover, hypothalamic N/OFQ peptide content was not influenced by lack of β-endorphin in the mutant mice model. Taken together these results suggest that changes in hypothalamic N/OFQ peptide levels in response to stress are not modulated by β-endorphin.

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Figure 5

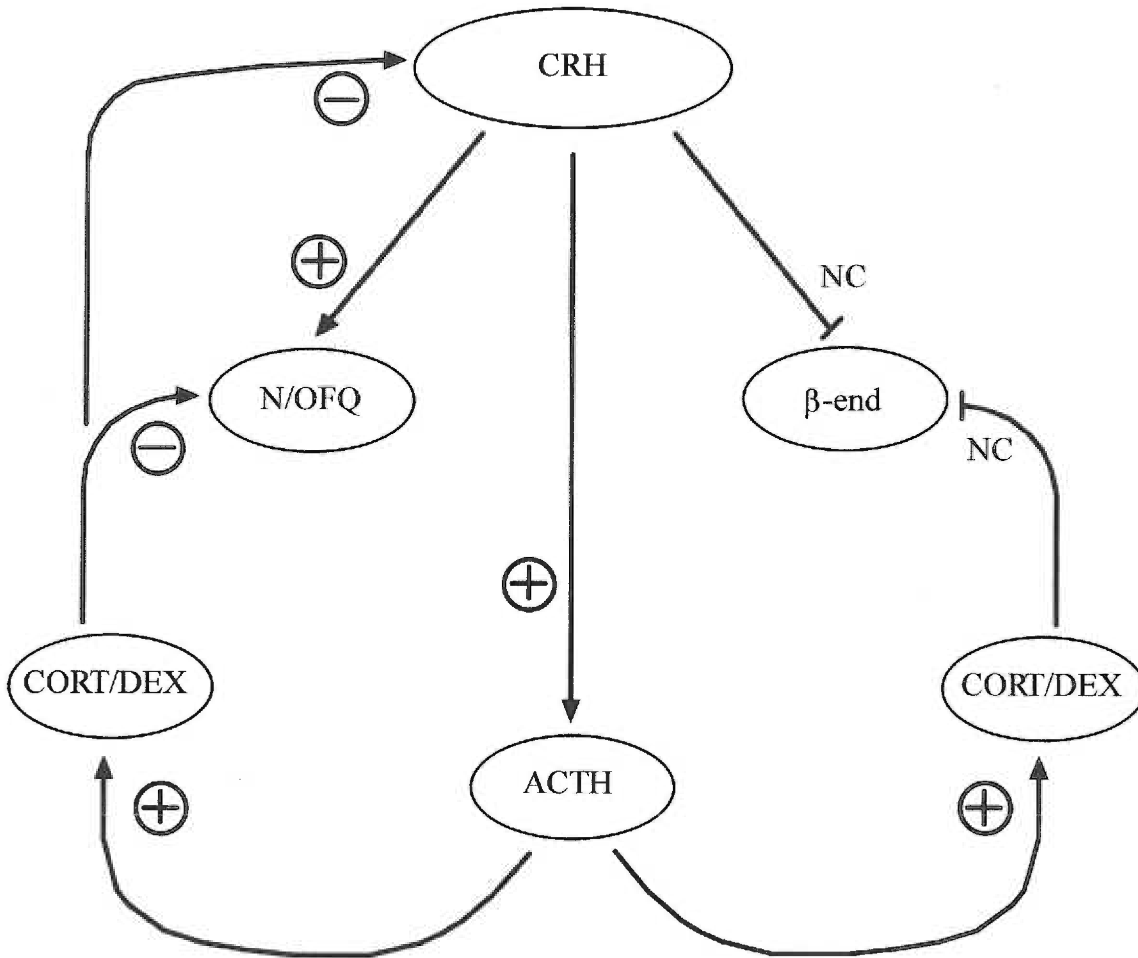

Figure 5. A model for stress response mediated changes to hypothalamic N/OFQ peptide content. CORT = glucocorticoids, β-end = β-endorphin, + = positive effect, - = negative feedback, NC = no change, DEX = dexamethasone.

Chapter 4

Integrity of Tritiated Orphanin FQ/Nociceptin: Implications for Establishing a Reliable Binding Assay

Integrity of Tritiated Orphanin FQ/Nociceptin: Implications for Establishing a Reliable Binding Assay

Denise Irene Quigley[a], Seksiri Arttamangkul[b], Richard Gordon Allen[c], David Kilgore Grandy[b,*]

[a]Department of Molecular and Medical Genetics, [b]Department of Physiology and Pharmacology, [c]Center for Research on Occupational and Environmental Toxicology, Oregon Health Sciences University, Portland, Oregon 97201, USA

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Corresponding author:
Dr. David K. Grandy
Department of Physiology and Pharmacology, L-334
Oregon Health Sciences University
3181 SW Sam Jackson Park Rd.
Portland, OR 97201 USA
phone: (503) 494-4671
FAX: (503) 494-4352
E-mail: grandyd@OHSU.edu

Running title: Integrity of [^{3}H] OFQ/N

Abstract

In the course of establishing a reliable and reproducible binding assay for the orphanin FQ/nociceptin (OFQ/N) ligand-receptor system we used reversed phase-high performance liquid chromatography (RP-HPLC) to monitor the integrity of [^{3}H] OFQ/N obtained from three different manufacturers. This means of analysis revealed that the stability of [^{3}H] OFQ/N during storage varied considerably depending on the manufacturer. Furthermore, the integrity of [^{3}H] OFQ/N was significantly compromised in the presence of COS-7 cell membranes. Interestingly, if the peptide was added to COS-7 membranes after they had been exposed to low pH it remained intact, suggesting that the peptide's breakdown during binding is, in part, enzymatically mediated. Although a variety of protease inhibitors were tested, none proved completely effective at protecting the tritiated peptide. The intention of the studies presented here was to evaluate OFQ/N binding components, namely the available [^{3}H] OFQ/N ligands, in an effort to standardize the binding conditions for this receptor ligand system. Consequently, this study underscores the importance of monitoring the integrity of the trace ligand being used in a given binding assay.

Keywords orphanin FQ/nociceptin; radioligand binding; nociceptin/OFQ receptor; peptide degradation

Introduction

The 17 amino acid peptide orphanin FQ/nociceptin (OFQ/N) (Fig. 1) is homologous to dynorphin A. It binds with high affinity to the orphan G-protein coupled receptor, N/OFQ receptor (NOR), which shares sequence homology to the μ, δ, and κ opioid receptors but this binding is insensitive to naloxone (Bunzow *et al.*, 1994; Meunier *et al.*, 1995; Reinscheid *et al.*, 1995). As with the opioid receptors, activation of the NOR mediates the inhibition of adenylyl cyclase and modulates K^+ and Ca^{2+} channel function. OFQ/N shows negligible affinity for the opioid receptors and the NOR does not appreciably bind other opioid ligands, suggesting a distinct non-opioid pharmacology. The OFQ/N peptide/receptor system has been implicated in numerous behaviors related to analgesia, stress, feeding, circadian rhythm and addiction, and in many situations is thought to possess anti-opioid properties (Darland *et al.*, 1998; Harrison and Grandy, 2000; Henderson and McKnight, 1998).

The pharmacology of the NOR has been investigated by several groups. However, upon comparison it is evident that there is considerable variability among the reported affinities of NOR for OFQ/N with K_d values that range from 20 pM to greater than 5 nM (Albrecht *et al.*, 1998; Dooley and Houghten, 1996; Makman *et al.*, 1997). In the interest of optimizing the binding conditions for OFQ/N to the NOR we evaluated tritiated peptide obtained from three commercial sources. The OFQ/N peptide is known to be cleaved *in vivo* and *in vitro* at its two dibasic (RK) enzyme cleavage sites (Sandin *et al.*, 1999; Suder *et al.*, 1999; Vlaskovska *et al.*, 1999). Some of the resultant fragments exhibit low binding affinity for the NOR and do not elicit the behavioral effects as does the intact OFQ/N peptide (Sandin *et al.*, 1999; Suder *et al.*, 1999; Vlaskovska *et al.*, 1999). Therefore, in the course of trying to establish a reliable binding assay we became concerned about the stability of commercially prepared OFQ/N during storage and binding. Here we

Figure 1

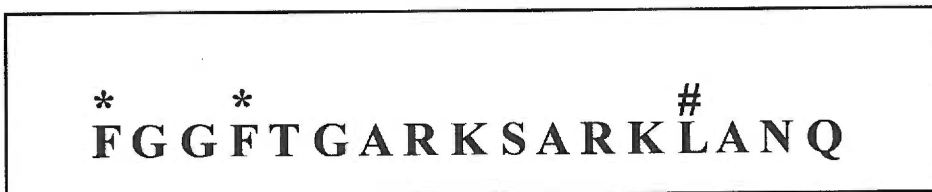

Figure 1. Amino acid sequence and location of tritium label for the MPS and Phoenix Pharmaceuticals peptide (*) and the Amersham Pharmacia peptide (#).

present the results of our analyses that indicate the inherent stability of [^{3}H] OFQ/N obtained from various commercial sources differs considerably and that [^{3}H] OFQ/N is degraded during the binding reaction.

Materials and Methods

Peptides and Chemicals

[^{3}H] OFQ/N was obtained from Multiple Peptide Systems (MPS, San Diego, CA), Phoenix Pharmaceuticals (Mountain View, CA) and Amersham Pharmacia (Piscataway, NJ) (Fig. 1) and upon receipt was aliquoted and stored at either -80° C (MPS) or 4° C (Phoenix and Amersham). The Amersham Pharmacia peptide was stored in a cocktail of protease inhibitors: 100 μg/ml bacitracin, 10 μg/ml leupeptin and 4 μg/ml chymostatin. Unlabeled OFQ/N was obtained as lyophilyzed powder from MPS, resuspended in 10% acetic acid and 1 mM aliquots stored at -20° C. RP-HPLC solvents, HPLC grade water, acetonitrile and triflouroacetic acid (TFA) were manufactured by Mallinckrodt. The RP-HPLC column was a Vydac C4 (5 μm, 300 Angstrom, 4.6x250 mm) obtained from the Separations Group (Hersperia, CA). GF/B glass fiber filters were manufactured by Schleicher and Schuell. All tissue culture reagents were purchased from Life Technologies. All other reagents were obtained from Sigma.

Cell Culture and Transfections

COS-7 cells were maintained at 37° C in 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum. The mouse OFQ/nociceptin receptor (mNOR) cDNA was cloned into the expression vector pcDNA 3.1 (Invitrogen) and transiently expressed in COS-7 cells following the calcium phosphate mediated method of

transfection. Briefly, COS-7 cells were grown to 40-60% confluence, exposed to 30 μg of construct or empty vector per 15 cm plate, incubated at 3% CO_2 four hours and then treated with 1x HBS (140 mM NaCl, 25 mM Hepes acid, 750 μM Na_2HPO_4, pH 7.05) containing 15% glycerol for 3 min. Cell membranes were harvested 48 hrs post transfection.

Membrane Preparation and Incubation Conditions

Radioligand stability studies were carried out on membranes prepared from COS-7 cells that had been transfected with the mNOR construct or pcDNA vector alone. Cells were harvested by manual scraping from 15 cm plates (Nunc) in 50 mM Tris-Cl buffer pH 7.4 followed by manual homogenization. Cellular membranes were isolated by centrifugation at 35,000 x g for 20 min at 4° C. Following centrifugation the Tris buffer was removed and membrane pellets were stored at -80° C. The membrane pellets were thawed, re-homogenized in 2-3 ml 50 mM Tris buffer pH 7.4 and protein concentrations determined by the method of Lowry (Lowry *et al.*, 1951). Membranes (75 μg in 100 μl Tris buffer), [^{3}H] OFQ/N and selected protease inhibitors were incubated at 25° C for 60 min (except for the time course study) in Tris buffer containing 100 μg/ml bacitracin and 1 mg/ml bovine serum albumin (BSA) in a final volume of 1 ml, the same conditions used for radioligand binding. For the time course studies samples were incubated for 0, 15, 30 or 60 min.

Radioligand Binding

For binding, membranes (75 μg in 100 μl Tris buffer) and various concentrations of [^{3}H] OFQ/N were incubated at 25° C for 60 min with and without unlabeled OFQ/N in 50 mM Tris buffer containing 100 μg/ml bacitracin and 1 mg/ml bovine serum albumin (BSA) in a final volume of 1 ml. Non-specific binding was determined in the presence of 1 μM unlabeled OFQ/N. Total binding and non-specific binding were determined in duplicate

for 12 concentrations of [^{3}H] OFQ/N within each assay. A Brandell multi-channel harvester was used to separate bound from free [^{3}H] OFQ/N by rapid filtration of the binding reactions through GF/B glass fiber filters that had been pretreated with 0.2% polyethyleneimine (PEI) for 60 min at 4° C. Filters were washed two times in the harvester with 2 ml of ice-cold 50 mM Tris-buffer pH 7.4. After washing the filter discs were punched out into scintillation vials and incubated overnight at room temperature on a rotating shaker in 3.5 ml of scintillation fluid (Fisher) then counted on a β–counter for 1 min. Data were analyzed using Microsoft Excel™ and curves fit using Inplot™ software. For post-binding RP-HPLC analysis an additional total binding tube containing [^{3}H] OFQ/N at a final concentration of 500 pM (172,000 dpm) was incubated along with the other assay tubes.

Membrane Pre-treatment with Protease and Proteosome Inhibitors

Untransfected COS-7 cell membranes (75 μg) were incubated in 0.1% TFA or boiled 10 min then incubated in 0.1% TFA (to 900 μl) then 100 μl (10,000 dpm) [^{3}H] OFQ/N (Amersham Pharmacia) was added and incubated as described. Membranes were collected from COS-7 cells transiently transfected with the mNOR and 75 μg boiled for 10 min then incubated with 10,000 dpm of [^{3}H] OFQ/N in Tris buffer as described. The Sigma Mammalian Tissue Protease Inhibitor Cocktail® (cat. # P 8340) containing AEBSF ([4-(2-aminoethyl)- benzene- sulfonylfluoride] hydrocloride), a PMSF alternative, aprotinin, bestatin, E-64, leupeptin and pepstatin A was diluted 1:10 and incubated with 75 μg of COS-7 mNOR membranes as described above. Amastatin, captopril and phosphoramidon, each at a final concentration of 1 μM, were incubated in various combinations: amastatin, captopril, or phosphoramidon alone, amastatin and captopril, amastatin and phosphoramidon, captopril and phosphoramindon or amastatin, phosphoramindon and captopril; with 75 μg of COS-7 mNOR membranes as described above. The proteosome

inhibitor N-Acetyl-Leu-Leu-Norleucinal (Calpain Inhibitor I) was incubated at a final concentration of 50 μM with 75 μg of COS-7 mNOR membranes as described above.

Reversed-Phase High Performance Liquid Chromatography (RP-HPLC)

For RP-HPLC analysis of the stock peptides 3-5 μg unlabeled OFQ/N and [^{3}H] OFQ/N, were diluted in 250 μl 0.1% trifluoroacetic acid (TFA) and injected into a Waters (Milford, NJ) RP-HPLC system connected to a C4 Vydac column. For RP-HPLC analysis of [^{3}H] OFQ/N incubated with membranes, samples were centrifuged 5 min at high speed in a table top centrifuge (Tomy) to remove membranes following incubation and the supernatant was then loaded onto the RP-HPLC column. Unlabeled OFQ samples were measured by absorbance at 214 nm. Labeled and unlabeled fractions were separated over 80 min in a linear gradient of 10-45% acetonitrile in 0.1% TFA at a flow rate of 1 ml/min. 500 μl of the resulting fractions were counted on a β–counter and the data were analyzed using Cricket Graph™ software. To determine the relative amount of degraded [3H] OFQ/N by RP-HPLC analysis, counts of radioactivity contained in fractions were compared to total input counts. In all cases, greater than 85% of input radioactivity could be accounted for in the resultant RP-HPLC fractions.

Mass Spectrometry

LC-MS analysis was performed on a Perkin Elmer/Sciex API III Ion spray triple quadruple mass spectrophotometer with an ion spray potential of 5000 V. The HPLC column was a 0.32 mm Luna C18 column (5 μm, 100 angstrom, 0.32x150 mm). All peptides were eluted within 15 min using a gradient of 5-50% acetonitrile in 0.1% acetic acid in 30 min at a flow rate of 5 μl/min. The orifice potential was 80-100 V. Molecular weights were calculated from the observed distributions of multiply charged ions using Automated HyperMass (Perkin Elmer/Sciex).

LC/MS analysis was performed on crude samples containing unlabeled OFQ/N peptide degraded in the presence of COS-7 cell membranes. In order to generate a sufficient quantity of degraded OFQ/N products to be detected by LC/MS 50 μg of unlabeled OFQ/N was incubated with 75 μg COS-7 cell membranes and the incubation time was extended to at least 16 hr at room temperature. In preparation for LC/MS, samples were centrifuged at 15,000 RPM in a table top centrifuge (Tomy) for 5 min to remove cellular material and supernatants transferred to a clean tube. Individual RP-HPLC fractions were also analyzed by LC/MS. Degraded OFQ/N peptide products, prepared as described above, were injected into a Vydac C4 RP-HPLC column and fractionated using a Waters RP-HPLC system with a linear gradient of 10-45% acetonitrile in 0.1% TFA in 80 min with a flow rate of 1 ml/min. Peptides were detected at 214 nm. RP-HPLC fractions were collected and OFQ/N peptide content was quantitated by radioimmunoassay as described previously (Quigley *et al.*, 1998). A sample containing only COS-7 cell membranes incubated and centrifuged as described and a sample containing only stock OFQ/N peptide were analyzed by LC/MS as controls.

Results

Labile Nature of [^{3}H] OFQ/N peptide

Using RP-HPLC and an OFQ/N radioimmunoassay (RIA), we previously established (Quigley *et al.*, 1998) that the unlabeled OFQ/N peptide (MPS) elutes from the column primarily in fraction 31 (at 31 min), (Fig. 2A). Due to lack of specific binding in multiple binding experiments conducted with the [^{3}H] OFQ/N peptide received from Multiple Peptides Systems (MPS), the integrity of the peptide became suspect. RP-HPLC analysis conducted on our stock revealed radioactivity in fractions that eluted from the column at 5-6, at 10-11 and at 31 min, suggesting degradation of the peptide had occurred

Figure 2

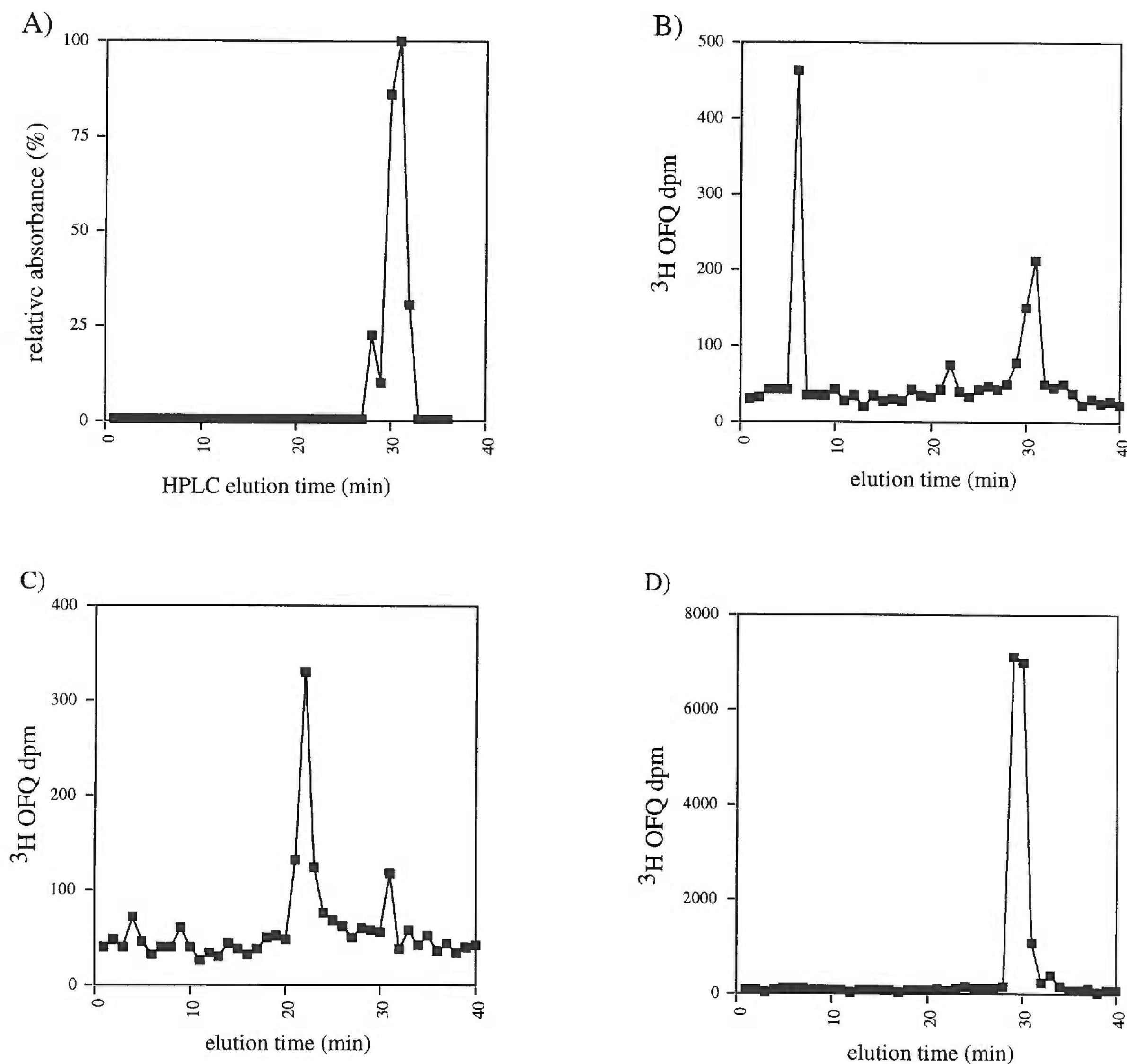

Figure 2. RP-HPLC profiles of stock OFQ/N (A) and [^{3}H] OFQ/N peptides from Multiple Peptide Systems (B), from Phoenix Pharmaceuticals (C) and from Amersham Pharmacia (D). For each [^{3}H] OFQ/N RP-HPLC trace approximately 48% (B), 17% (C) and 100% (D) of the input radioactivity eluted from the column at 31 min, representing the relative amount of [^{3}H] OFQ/N that remained in tact.

during shipping and/or handling (data not shown). When a second stock of [^{3}H] OFQ/N peptide was received from MPS, it was aliquoted and immediately frozen at -80° C (at the suggestion of the manufacturer). Within 24 hrs of receipt RP-HPLC analysis was carried out on this new peptide. Greater than 50% of the radioactivity eluted from the column at 5-6 min (fig 2B), which may represent either degraded peptide or insufficient purification of the peptide after tritiation. Unlabeled OFQ/N peptide loaded simultaneously on to the RP-HPLC column eluted at 31 min, as expected.

[^{3}H] OFQ/N peptide was also obtained from Phoenix Pharmaceuticals. When it was received the [^{3}H] OFQ/N peptide was immediately aliquoted and stored at 4° C per the manufacturers recommendation. Within 24 hrs of receipt the [^{3}H] OFQ/N peptide and unlabeled OFQ/N were run on our RP-HPLC column and greater than 95% of the input radioactivity co-eluted from the column at the same time as the unlabeled OFQ/N peptide (31 min) indicating that at the time of receipt the [^{3}H] OFQ/N peptide was intact. However, when binding experiments were conducted with the Phoenix [^{3}H] OFQ/N peptide no specific binding to NOR was observed (data not shown). When RP-HPLC analysis of the stock peptide was repeated one month following its receipt the majority of the radioactivity eluted from the column at 20-22 min (Fig. 2C) indicating that the [^{3}H] OFQ/N peptide was unstable when stored under these conditions.

[^{3}H] OFQ/N peptide was also obtained from Amersham Pharmacia and stored at 4° C in a cocktail of protease inhibitors including bacitracin, leupeptin and chymostatin (see methods). RP-HPLC analysis of the Amersham Pharmacia peptide indicated that the radioactivity eluted from the column at 31 min (Fig. 2D), consistent with the elution time of intact OFQ/N peptide. Periodic RP-HPLC analyses of the stock peptide gave identical results, suggesting that the Amersham peptide was stable under the recommended storage conditions. Interestingly, an aliquot of the peptide stored in the absence of protease inhibitors was also stable according to our RP-HPLC analysis, indicating that the [^{3}H]

OFQ/N peptide received from Amersham Pharmacia was inherently more stable than the [^{3}H] OFQ/N peptide received from either MPS or Phoenix Pharmaceuticals.

Integrity of [^{3}H] OFQ/N peptide following the binding assay

Given that our RP-HPLC analysis revealed that the [^{3}H] OFQ/N peptide received from Amersham Pharmacia was consistently the most stable over time in storage, it was used in our subsequent binding studies. Figure 3A is a representative plot of a post-binding RP-HPLC analysis. This RP-HPLC plot represents the [^{3}H] OFQ/N that remained in the supernatant after the membranes were pelleted out after binding. Consequently, this profile does not represent the [^{3}H] OFQ/N that was specifically bound to NOR or trapped non-specifically in the membrane pellet. Indeed, the calculated dpm of [^{3}H] OFQ/N peptide that must be associated with the pellet in this experiment is sufficient to saturate NOR sites as determined by the saturation binding curve (Fig. 3B). This data suggests that NOR binding is high affinity and rapid such that, reproducible saturable binding can be obtained (K_d = 28.3 pM, comparable to other published values) in spite of the significant amount of [^{3}H] OFQ/N peptide degradation that occurs over time (Fig. 3B).

An RP-HPLC time-course experiment was carried out next to examine the rate of OFQ/N degradation during incubation. At time zero and 15 min approximately 15% and 85%, respectively, of the input [^{3}H] OFQ/N eluted at 6-7 min. After 30 min of incubation virtually all of the input [^{3}H] OFQ/N eluted at 6-7 min, suggesting that a negligible amount of the radiolabeled peptide remains intact (Fig. 4).

Integrity of [^{3}H] OFQ/N in the Presence of Protease and Proteosome Inhibitors

Since the radioactivity consistently eluted from the RP-HPLC column in distinct fractions following incubation with membranes, we reasoned that the [^{3}H] OFQ/N peptide was being enzymatically processed, perhaps at the two RK dibasic cleavage sites

Figure 3

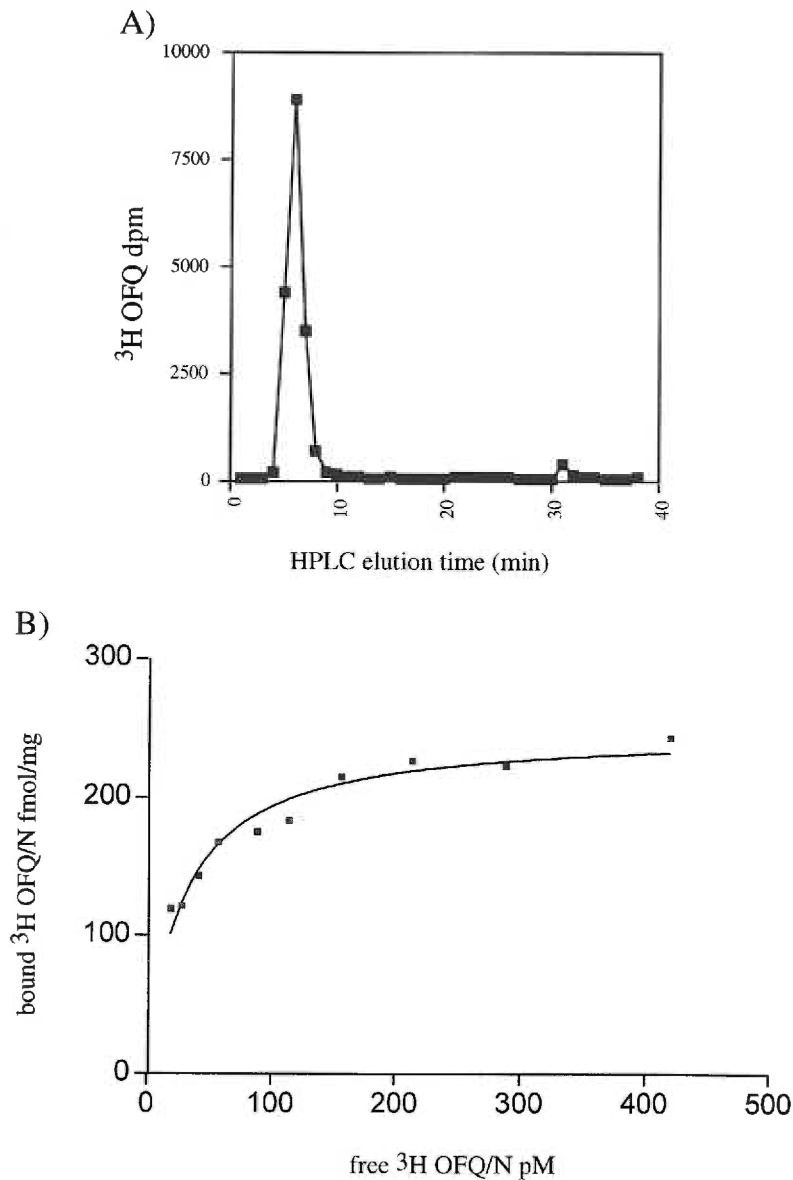

Figure 3. Post-binding RP-HPLC analysis (A). An additional tube was prepared as described for binding with [^{3}H] OFQ/N (Amersham Pharmacia) at a final concentration of 500 pM and incubated along with the binding samples. Following incubation the sample was centrifuged to remove cellular material and the supernatant fractionated by RP-HPLC. The peak of radioactivity eluting from the column at 5-6 minutes represents approximately 85% of the input radioactivity (before membranes were pelleted out), suggesting that about 15% of the input radioactivity remained intact or was contained in the membrane pellet. (B) Saturation binding curve with Amersham Pharmacia [^{3}H] OFQ/N at a final concentration of 20-500 pM, incubated at 25° C for 1 hr with 75 μg of membranes prepared from COS-7 cells transiently expressing the mouse NOR. K_d = 28.3 pM and Bmax = 248.4 fmol/mg.

Figure 4

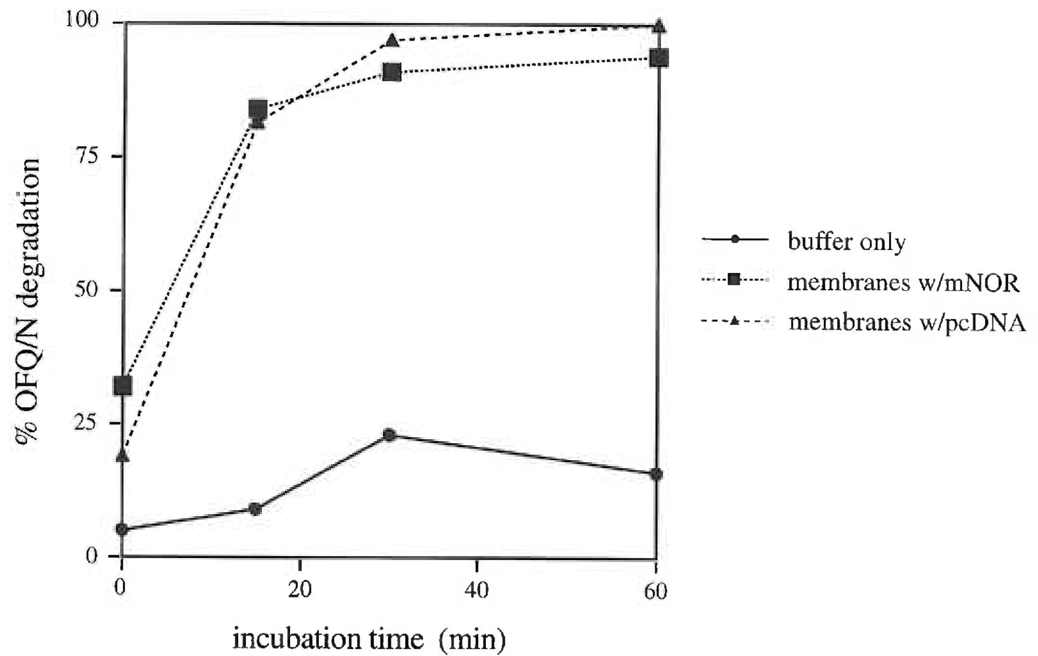

Figure 4. Time course of [^{3}H] OFQ/N degradation. [^{3}H] OFQ/N from Amersham Pharmacia was incubated with 75 μg of membranes prepared from COS-7 cells transiently expressing the mouse NOR in pcDNA3.1 or pcDNA3.1 vector alone. Samples were incubated at 25° C for 0, 15, 30 or 60 min, centrifuged to pellet the membranes and then fractionated by RP-HPLC analysis. Individual fractions were then counted for the presence of [^{3}H].

contained within the 17 amino acid peptide. When [^{3}H] OFQ/N peptide was incubated with membranes in the presence of 0.1% TFA or the membranes were boiled then incubated in 0.1% TFA it eluted from the RP-HPLC column at 31 min, the expected time determined for intact OFQ/N peptide (Fig. 5A). Boiling of COS-7 mNOR membranes for 10 min prior to incubation with [^{3}H] OFQ/N in Tris buffer resulted in [^{3}H] OFQ/N eluting from the column primarily at 5 and 31 min (Fig. 5B). In an effort to determine if the degradation of OFQ/N could be blocked by protease inhibitors, [^{3}H] OFQ/N peptide and COS-7 mNOR membranes were incubated in the presence of the Sigma Mammalian Protease Inhibitor Cocktail®. This treatment resulted in a reduction of peptide degradation compared to samples incubated in the absence of protease inhibitors (Fig. 5C), such that approximately 65% of the input [^{3}H] OFQ/N remained intact. None of the protease inhibitors: amastatin, phosphoramidon or captopril alone or in combination with one another significantly inhibited the degradation of the Amersham [^{3}H] OFQ/N peptide. Figure 5D is a representative trace of RP-HPLC results obtained when OFQ/N was incubated with amastatin, phosphoramidon and captopril. In order to determine whether the degradation of [^{3}H] OFQ/N was due to proteosome action, [^{3}H] OFQ/N peptide and COS-7 mNOR membranes were incubated in the presence of the proteosome inhibitor N-Acetyl-Leu-Leu-Norleucinal. The RP-HPLC results indicated no significant reduction in [^{3}H] OFQ/N degradation, suggesting that the degradation is most likely not proteosome mediated (data not shown).

Biotransformation of OFQ/N by Enzymatic Activity in COS-7 Cells

Since the commonly used protease inhibitors failed to inhibit the degradation of the OFQ/N peptide mediated by COS-7 cell membranes. We utilized mass spectrometry analysis of unlabeled OFQ/N in an attempt to determine the exact nature of the cleavage products in the hope of identifying the sequences recognized by the COS-7 enzyme(s)

Figure 5

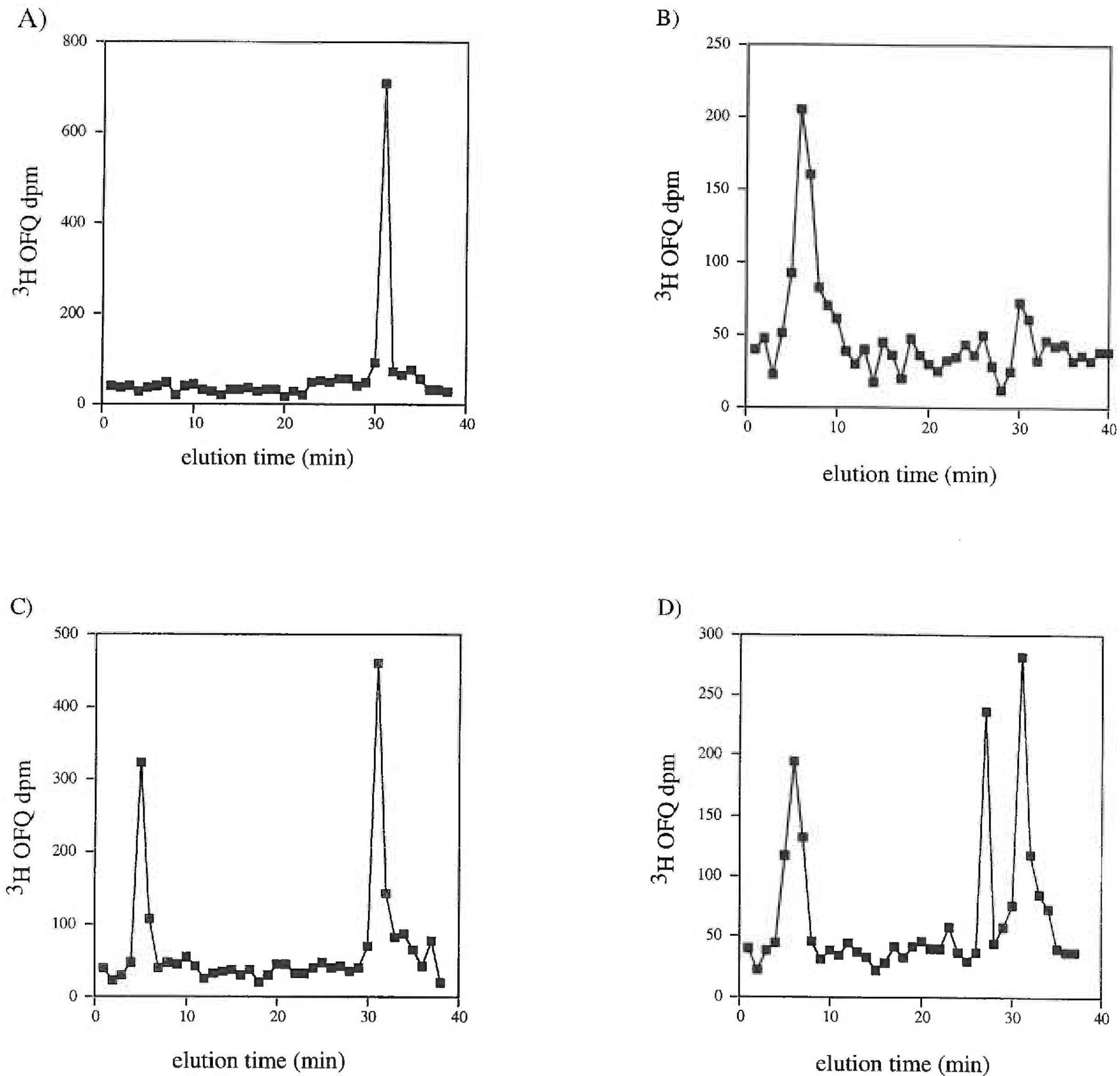

Figure 5. Effect of membrane pre-treatment or protease inhibitors on [^{3}H] OFQ/N stability. (A) 75 µg of membranes prepared from untransfected COS-7 cells were incubated in 0.1% TFA or boiled for 10 min and then incubated in 0.1% TFA or (B) boiled for 10 min only. The membranes were incubated with [^{3}H] OFQ/N from Amersham Pharmacia for 1 hr at 25° C, centrifuged and supernatants were fractionated by RP-HPLC. (C) Sigma Protease Inhibitor Cocktail® at a 1:10 dilution or (D) protease inhibitors amastatin, phosphoramidon, and captopril each at a final concentration of 1 µM were incubated with 75 µg of membranes prepared from COS-7 cells that transiently expressed the mouse NOR and [^{3}H] OFQ/N from Amersham Pharmacia as described, samples centrifuged and supernatants fractionated by RP-HPLC. For each [^{3}H] OFQ/N RP-HPLC trace approximately 97% (A), 18% (B), 65% (C) and 32% (D) of the input radioactivity eluted from the column at 31 min which represents the [^{3}H] OFQ/N peptide that remained in tact.

responsible for the observed degradation. Mass spectrometry analysis revealed that the major OFQ/N degradation products produced during incubation with COS-7 membranes were the amino terminal fragments OFQ/N $^{1-13}$, OFQ/N $^{1-14}$, and OFQ/N $^{1-15}$ (Fig. 6). RP-HPLC revealed that OFQ/N $^{1-17}$ (retention time 10.20 min) was metabolized at the C-terminus yielding the OFQ/N $^{1-15}$ fragment (retention time 9.90 min), the OFQ/N $^{1-14}$ fragment (retention time 9.75 min) and the OFQ/N $^{1-13}$ fragment (retention time 8.29 min). Additional peaks were also observed. The first of these (retention time 1.21 min) had a mass that could not be determined. The other peaks contained peptides of mass 1057.3 (retention time 2.29) and 1299.6 (retention time 4.92 min). The crude sample of degraded OFQ/N products was fractionated by RP-HPLC and individual fractions were analyzed by LC/MS to confirm presence of the N-terminal OFQ/N products described above. No other masses could be confirmed by LC/MS of individual RP-HPLC fractions (data not shown). Moreover, results of radioimmunoassay (RIA) of individual RP-HPLC fractions for OFQ/N supported the results found by LC/MS (data not shown).

Discussion

Several groups have reported affinity constants for [^{3}H] OFQ/N binding to NOR, however the wide range of reported values suggested to us that the stability of the ligand might be an issue. The [^{3}H] OFQ/N obtained from MPS and Phoenix Pharmaceuticals appeared to be labile during storage. These [^{3}H] OFQ/N peptides are labeled on the phenylalanine residues at positions 1 and 4. In contrast, our analysis revealed that the Amersham Pharmacia peptide, labeled on the leucine residue at position 14 (Fig. 1), is stable during storage for up to two months.

Recently, there have been a few reports describing the proteolytic cleavage of OFQ/N $^{1-17}$ and the putative functional significance of the resulting products, both *in vitro* and *in vivo*. In mouse cortical brain slices OFQ/N can be metabolized by endopeptidase

Figure 6

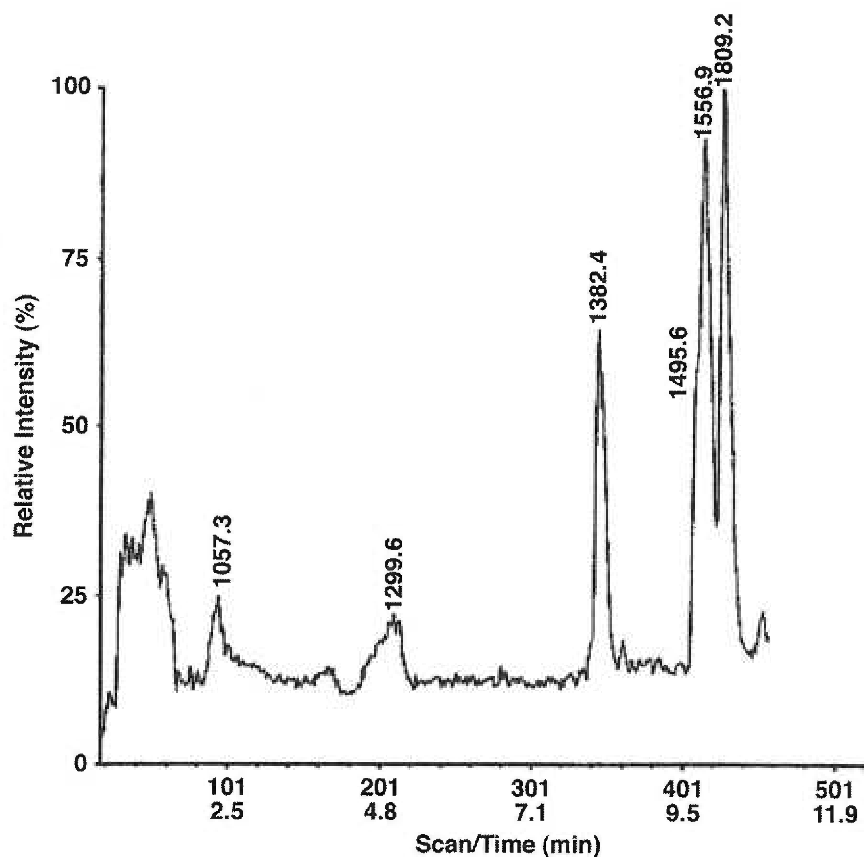

Figure 6. Mass chromatogram of OFQ/N biotransformation products. Labeled peaks represent multiply charged ions corresponding to masses 1057.3, 1299.6, 1382.4 (OFQ/N $^{1\text{-}13}$), 1495.6 (OFQ/N $^{1\text{-}14}$), 1566.9 (OFQ/N $^{1\text{-}15}$) and 1809.2 (OFQ/N $^{1\text{-}17}$). The intensity scale is shown on the left side of the chromatogram and is expressed as the % of the value of the highest peak.

24.15 and aminopeptidase N into OFQ/N $^{1-7}$, OFQ/N $^{12-17}$, OFQ/N $^{13-17}$ and OFQ/N $^{2-17}$ (12). In the rat spinal cord OFQ/N is truncated through the action of a serine endopeptidase to OFQ/N $^{1-11}$ and ultimately to OFQ/N $^{1-6}$ (Suder *et al.*, 1999). Vlaskovska *et al.* reported that OFQ/N was cleaved into OFQ/N $^{1-9}$ and OFQ/N $^{1-13}$ in the presence of cultured cell lines. This process was presumably mediated by a metallosensitive endopeptidase, since it could be inhibited by Hg^{2+}, Cu^{2+}, Zn^{2+} PMSF and EDTA (Vlaskovska *et al.*, 1999). In the rat hippocampus OFQ/N is metabolized first to OFQ/N $^{1-13}$ and OFQ/N $^{14-17}$ and then OFQ/N $^{1-13}$ is further metabolized to OFQ/N $^{1-9}$ and OFQ/N $^{10-13}$ (Sandin *et al.*, 1999).

Hiranuma *et al.* reported that the combination of amastatin, captopril and phosphoramidon was sufficient to prevent the enzymatic degradation of dynorphin-(1-8) in the presence of membranes prepared from guinea-pig ileum or striatum (Hiranuma *et al.*, 1998). Considering the similarity between OFQ/N and dynorphin-(1-8) this combination of inhibitors to an aminopeptidase, a dipeptidyl carboxypeptidase, and endopeptidase-24.11, was expected to completely protect OFQ/N $^{1-17}$ from cleavage in vitro. It is intriguing that the degradation of OFQ/N was completely blocked in a low pH environment whereas boiling of the COS-7 cell membranes did not completely prevent OFQ/N breakdown, suggesting the putative enzyme(s) mediating the peptides degradation is relatively heat-stable but pH sensitive.

Our RP-HPLC analysis led us to explore the nature of the $[^3H]$ OFQ/N degradation products by mass spectrometry in the hope of discovering a clue to the identity of the enzyme(s) responsible. To obtain a sufficient quantity of degradation product for mass spectrometry we chose to use the unlabeled OFQ/N rather than $[^3H]$ OFQ/N. Our mass spectrometry investigation revealed that when the peptide is incubated with COS-7 membranes the majority of the cleavage appears to be mediated by a carboxypeptidase that produces the fragments OFQ/N $^{1-15}$, OFQ/N $^{1-14}$ and OFQ/N $^{1-13}$. Alternatively, the OFQ/N $^{1-13}$ product may result from an endopeptidase cleaving the peptide between residues

13 and 14. Unfortunately, the major [^{3}H] OFQ/N degradation product(s) that eluted in fractions 5-6 could not be identified by mass spectrometry of the unlabeled OFQ/N. Interestingly, OFQ/N $^{1-13}$ does not appear to be cleaved further either by a carboxypeptidase yielding OFQ/N $^{1-12}$, OFQ/N $^{1-11}$, etc., or by an endopeptidase yielding OFQ/N $^{1-9}$. If the OFQ/N cleavage yielding OFQ/N $^{1-13}$ were in fact mediated by an endopeptidase, this finding suggests that the endopeptidase responsible for this cleavage in COS-7 cell membranes differs from that reported by Sandin *et al.* in the rat hippocampus (Sandin *et al.*, 1999). The lack of further cleavage products suggests that the OFQ/N $^{1-13}$ is no longer a good substrate for proteases associated with COS-7 cell membranes.

If OFQ/N $^{1-17}$ was originally labeled at leucine 14 then the degraded product, OFQ/N$^{1-13}$, would no longer contain the tritium label. OFQ/N $^{1-13}$ has been shown to have moderate binding affinity for the NOR (Guerrini *et al.*, 1997). Therefore, the [^{3}H] OFQ/N degradation product OFQ/N $^{1-13}$ might be expected to compete with [^{3}H] OFQ/N for the NOR during the binding experiment. This competition for the binding site may be, in part, responsible for some of the variability in reported binding affinities. Dooley and Houghten have reported that of the truncated OFQ/N peptides, OFQ/N $^{1-13}$ amide was the shortest fragment that retained affinity for the NOR in rat brain homogenates (Dooley and Houghten, 1996). In contrast, non-amidated OFQ/N $^{1-13}$ has relatively low affinity for the NOR (Guerrini *et al.*, 1997). Furthermore, OFQ/N $^{1-13}$ amide exhibits full biological activity in the mouse vas deferens. Consequently, C-terminal amidation of OFQ/N-derived peptides appears to be necessary for their protection from proteolytic degradation (Guerrini *et al.*, 1997). In spite of the activity of OFQ/N $^{1-13}$ -NH_2 in the mouse vas deferens, Sandin *et al.* reported that OFQ/N $^{1-13}$ had no effect on spatial cognitive function in the Morris water maze (Sandin *et al.*, 1999). This data, taken together with our studies of the stability of [^{3}H] OFQ/N, suggest that an amidated, C-terminally truncated OFQ/N, perhaps OFQ/N $^{1-14}$

retaining the leucine 14 for tritium label, may be a more suitable ligand than OFQ/N^{1-17} for use in *in vitro* binding assays.

Of the peptides tested, we found that the $[^3H]$ OFQ/N obtained from Amersham Pharmacia, labeled at leucine 14, was the most stable during storage. Subsequent binding studies revealed that when this ligand was incubated in the presence of membranes prepared from untransfected COS-7 cells as well as those expressing the mNOR degradation of $[^3H]$ OFQ/N still occurred. This finding suggests that the degradation of $[^3H]$ OFQ/N is not mediated through the receptor binding process but by a cleavage process inherent to the COS-7 cell membranes themselves. It is interesting that the cleavage of OFQ/N observed in various tissues and cell lines results in discrete, tissue or cell line specific products (Sandin *et al.*, 1999; Suder *et al.*, 1999; Vlaskovska *et al.*, 1999). One can not dismiss the possibility that OFQ/N degradation products may possess biological activity at receptor sites other than the NOR. Therefore, it is especially important to monitor $[^3H]$ OFQ/N integrity in receptor binding experiments conducted in brain homogenates. $[^3H]$ OFQ/N background binding, presumably non-specific sticking of the peptide to tubes and GF/B filters, is another factor that may also account for some of the variability in binding results. Although inclusion of BSA and bacitracin in the binding appears to significantly reduce background, as with the other protease inhibitors tested here these additives had little or no effect on the degradation of $[^3H]$ OFQ/N during binding. Finally, in spite of the degradation of the ligand that occurred during the binding reaction, reproducible, saturable binding was still achieved using the $[^3H]$ OFQ/N obtained from Amersham Pharmacia and may be a reflection of the high affinity of the OFQ/N for NOR.

Acknowledgments

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Chapter 5

Effects of Point Mutations in the Nociceptin/Orphanin FQ Receptor on Receptor-Ligand Interactions

Effects of Point Mutations in the Nociceptin/Orphanin FQ Receptor on Receptor-Ligand Interactions

Denise I. Quigley[1], M. Germana Paterlini[2], James R. Bunzow[3] and David K. Grandy[3]

[1]Department of Molecular and Medical Genetics, [3]Department of Physiology and Pharmacology, Oregon Health Sciences University, Portland, OR 97201; [2]Department of Medicinal Chemistry, University of Minnesota, Minneapolis, MN 55455

Corresponding author:

David K. Grandy, Ph.D.
3181 SW Sam Jackson Park Rd.
Department of Physiology and Pharmacology, L-334
Oregon Health Sciences University
3181 SW Sam Jackson Park Rd.
Portland, OR 97201 USA
phone: (503) 494-4671
fax: (503) 494-4352
E-mail: grandyd@ohsu.edu

Running title: Nociceptin/orphanin FQ receptor mutagenesis

Abstract

In an effort to identify specific amino acid residues that may be involved in binding and activation of the mouse nociceptin/orphanin FQ receptor (mNOR), receptor mutants were created via site-directed mutagenesis. It was hypothesized that an aspartic acid (D107) residue in putative transmembrane domain 2 (TMD II) and an arginine (R299) residue in putative transmembrane domain 7 (TMD VII) form an interhelical salt bridge that constrains the mNOR binding site and holds the receptor in an inactive conformation. When agonist binds this putative salt bridge constraint would be disrupted resulting in the activation of mNOR. This proposed mechanism of mNOR activation is modeled after that of the rhodopsin and α_{1b} -adrenergic receptors. Site-directed mutagenesis of similar residues within these G protein-coupled receptors results in constitutively active receptors, presumably by disruption of a putative salt bridge linkage. To test whether mNOR is similarly activated three mutant forms of the receptor were created: D107 and R299 were changed to the uncharged residue alanine (A) individually (mNOR D107A, mNOR R299A) and simultaneously (mNOR D107A/R299A) in the receptor. The R299A form of the receptor expressed in COS-7 cells displayed increased binding affinity for N/OFQ compared with the wild-type receptor. In contrast, the D107A mutant and the D107A/R299A double mutant exhibited reduced binding affinity for N/OFQ. When expressed in HEK cells all of the mutations resulted in less potent N/OFQ mediated inhibition of adenylyl cyclase activity when compared to the wild-type receptor. Therefore, unlike the rhodopsin and α_{1b}-adrenergic receptor mutants, these mutant NORs do not appear to be constitutively active, suggesting that disruption of the putative salt bridge linkage between D107 and R299 is not sufficient for activation of NOR or that a salt bridge linkage does not exist between these two amino acid residues.

Key Words nociceptin/orphanin FQ receptor (NOR), Orphanin FQ, nociceptin, site directed mutagenesis, binding, cAMP

Introduction

Cloning of the classical μ-, δ- and κ- opioid receptors lead to the identification, by sequence homology, of a previously uncharacterized opioid-like receptor (Bunzow *et al.*; Fukuda, *et al.*; Mollereau *et al.*; Pan *et al.*; Wang *et al.*; Wick *et al.*, 1994). This orphan receptor fails to bind any known opiate compounds or opioid peptides with appreciable affinity (Bunzow *et al.*; Mollereau *et al.*, 1994). Using a functional assay two groups simultaneously identified the endogenous peptide agonist for this novel receptor. The ligand, a 17 amino acid neuropeptide, is termed orphanin FQ (Reinscheid *et al.*, 1995) since it activates the orphan receptor and nociceptin (Meunier *et al.*, 1995) due to its apparent hyperalgesic properties. Although the nociceptin/orphanin FQ (N/OFQ) peptide resembles other opioid peptides, particularly dynorphin A, it does not exhibit appreciable affinity for the μ-, δ- or κ - opioid receptors (MOR, DOR or KOR) (Reinscheid *et al.*, 1995). The primary function of N/OFQ is unknown, however, in contrast with the opioid peptides it does not appear to produce analgesia supraspinally. Indeed, N/OFQ possesses anti-opiate properties (Grisel *et al.*, 1996; Heinricher *et al.*, 1997; Mogil *et al.*, 1996a), impairs spatial learning (Sandin *et al.*, 1997), increases (Florin *et* al., 1996) or decreases (Devine *et al.*, 1996; Reinscheid *et al.*, 1995) locomotion depending on route of administration, affects feeding behavior (Pomonis *et al.*, 1996; Leventhal *et al.*, 1998; Candeletti *et al.*, 1998) and possesses anxiolytic properties (Jenck *et al.*, 1997).

In addition to the differences in behavioral effects, the N/OFQ peptide appears to differ from the opioid peptides in its structure-function relationship. For example, dynorphin A is thought to contain a "message" domain (the first five amino acids) which confers opioid activity and an "address" domain (the C-terminal residues) that is responsible for peptide potency and receptor selectivity (Chavkin and Goldstein, 1981). Mutagenesis studies of N/OFQ, including C-terminal truncation (Reinscheid *et al.*, 1996) and alanine scanning (Dooley and Houghten; Reinscheid *et* al., 1996) argue against a

"message-address" mode of binding at the nociceptin/orphanin FQ receptor (NOR). In a recent report Lapalu and colleagues examined the structure-activity relationship of chimeric N/OFQ/dynorphin A peptides at NOR and at the kappa opioid receptor (KOR). The results of these studies suggest that in spite of the sequence similarities between these two peptides and their respective receptors, N/OFQ and dynorphin A do indeed have different functional architectures (Lapalu *et al.*, 2000). Furthermore, an NOR/KOR chimera has been identified that binds and is activated by both N/OFQ and dynorphin A as well as the parent receptors respond to their respective ligands. These results suggest that N/OFQ and dynorphin A bind their receptors at different sites and activate via different mechanisms (Mollereau *et al.*, 1999).

The tertiary structure of the seven transmembrane spanning α helices that comprise G protein-coupled receptors (GPCRs) has been studied by several groups. Protein modeling suggests that GPCRs are composed of seven ideal α helices that are arranged in a counterclockwise fashion (1-7) as viewed from the extracellular surface. The helices 2,3,5,6 and 7 form the tightly packed inner core while helices 1 and 4 are most exposed to the plasma membrane (Alkorta and Loew, 1996; Strahs and Weinstein, 1997; Pogozheva *et al.*, 1998). Opioid receptor modeling predicts that amino acid residues in helices 2 and 7 participate in intrahelical hydrogen bonding which may indirectly affect ligand binding (Strahs and Weinstein, 1997; Pogozheva *et al.*, 1998). Furthermore, these hydrogen bonds may be involved in receptor activation. Replacement of amino acid residues involved in intrahelical hydrogen binding by residues or different size or hydrogen bonding capacity is expected to induce shifts in entire helices (Pogozheva, *et al.*, 1998). The signal transduction mechanism of GPCRs is thought to be mediated via a conformational change in the receptor transmitted by ligand binding (Strahs and Weinstein, 1997). Thus, GPCR activation may involve a conformational change induced by the disruption of intrahelical hydrogen bonds.

The mechanism of activation of the GPCR rhodopsin involves the disruption of a salt bridge that forms between the glutamic acid residue 113 (E113) in transmembrane

(TM) helix III and the lysine 296 (K296) residue in TM helix VII, which forms a Schiff's base with retinal (Robinson *et al.*, 1992). Light activates the rhodopsin receptor by inducing isomerization of the cis form of retinal to trans, disrupting the salt bridge between E113 - K296. Disruption of this salt bridge by site directed mutagenesis leads to a constitutively active conformation of the rhodopsin receptor (Robinson *et al.*, 1992). Activation of another well-studied G protein-coupled receptor, the α_{1b}-adrenergic receptor, appears to be mediated via the disruption of a similar salt bridge constraint. Site- directed mutagenesis of the homologous residues in the α_{1b}-adrenergic receptor, D125 and K331, resulted in constitutive activity as measured by production of soluble inositol phosphate (Porter *et al.*, 1996). These results suggest that the rhodopsin and α_{1b}-adrenergic receptors share a similar mechanism of activation that involves the disruption of an interhelical salt bridge constraint.

We have conducted protein modeling studies of the mNOR and determined that if TM helix 7 were an ideal α helix and TM helix 2 were an ideal helix with an end of helix motif then amino acid residues R299 and D107 would face the interior of the receptor. We propose that given their orientation, these amino acid residues may be involved in formation of a salt bridge constraint similar to that of the rhodopsin and α_{1b}-adrenergic receptors. To test the hypothesis that the mechanism of mNOR activation may be mediated via disruption of this putative salt bridge we mutated the D107 and R299 amino acid residues to alanine using site directed mutagenesis. Here we report characterization of the binding and functional coupling of the mutant mNORs D107A, R299A and the double mutant mNOR D107A/R299A. Our results indicate that mutation of these residues altered the affinity of N/OFQ for NOR. In contrast to the rhodopsin and α_{1b}-adrenergic receptors, the NOR mutant receptors do not appear to be constitutively active suggesting that the activation of NOR is not dependent on disruption of this putative salt bridge or that the D107 and R299 residues do not form an interhelical salt bridge linkage.

Materials and Methods

Peptides and Chemicals

[^{3}H] N/OFQ was obtained from Amersham Pharmacia (Piscataway, NJ) and upon receipt was aliquoted and stored in a cocktail of protease inhibitors: 100 μg/ml bacitracin, 10 μg/ml leupeptin and 4 μg/ml chymostatin at 4° C. Unlabeled N/OFQ was obtained as lyophilyzed powder from Multiple Peptide Systems (MPS), resuspended in 10% acetic acid and 1 mM aliquots stored at -20° C. GF/B glass fiber filters were manufactured by Schleicher and Schuell. All tissue culture reagents were purchased from Life Technologies. All other reagents were obtained from Sigma.

Site Directed Mutagenesis

Site directed mutagenesis was performed on the mouse nociceptin/OFQ receptor (mNOR) contained in the pBK-CMV vector (a generous gift from Dr. S. Nagalla) using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene). Briefly, fully overlapping PCR primers containing the mutation were utilized to amplify the entire pBK-CMV mNOR construct. Template DNA was digested with DpnI and Epicurian Coli® XL-1 Blue supercompetent cells transformed with the remaining PCR product. Plasmid DNA was isolated from selected bacterial colonies and the entire receptor sequenced to confirm mutation and to confirm that no other mutations were introduced in the receptor sequence by PCR. The mutated mNOR was later sub-cloned into the pcDNA 3.0 expression vector (Invitrogen).

Cell Culture and Transfections

COS-7 and human embryonic kidney (HEK) cells were maintained at 37° C in 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum. The wild type or the mutant mouse Nociceptin/Orphanin FQ receptor (mNOR) cDNAs, cloned into the expression vector pcDNA 3.0, were transiently expressed individually in COS-7 cells following the calcium phosphate mediated method of transfection. Briefly, COS-7 cells were grown to 40-60% confluence, exposed to 30 µg of construct or empty vector per 15 cm plate, incubated at 3% CO_2 4 hours and then treated with 1x HBS (140 mM NaCl, 25 mM Hepes acid, 750 µM Na_2HPO_4, pH 7.05) containing 15% glycerol for 3 min. Cell membranes were harvested 48 hrs post transfection.

For cAMP assays HEK cells were co-transfected with the wild type or mutant mNOR cDNAs and the human dopamine D_1 receptor (hD_1-R) contained in the pBC eukaryotic expression vector (Invitrogen) using Lipofectamine™ reagent (GibcoBRL). HEK cells were grown to 70-80% confluence, exposed to 15 µg each of the indicated mNOR construct and the hD_1-R construct in the presence of Lipofectamine™ reagent and incubated in 3% CO_2 4 hours in serum free DMEM. Cells were maintained in DMEM containing 10% fetal calf serum for 48 hours following transfection and cells harvested for the cAMP assays.

To determine transfection efficiency, cells were transfected as described above with 15 µg each hD1-R, mNOR and soluble eGFP (Clontech) in a CMV promoter containing expression vector. Approximately 48 hours following transfection cells were counted under phase contrast and fluorescence and the percentage of GFP expressing cells determined.

Membrane Preparation and Radioligand Binding

Transfected COS-7 cells were harvested by scraping in 50 mM Tris-Cl buffer pH 7.4. Membranes were prepared by manual homogenization in the Tris buffer and centrifugation at 35,000 x g for 20 minutes at 4° C. Following centrifugation Tris buffer was removed and membranes stored at -80° C until use. For binding, membrane pellets were manually homogenized in Tris buffer a second time and protein concentrations determined using the method of Lowry (Lowry *et* al., 1951). For binding, membranes (75 µg in 100 µl Tris buffer) and various concentrations of [^{3}H] N/OFQ were incubated at 25° C for 60 min with and without unlabeled N/OFQ in 50 mM Tris buffer containing 100 µg/ml bacitracin and 1 mg/ml bovine serum albumin (BSA) in a final volume of 1 ml. Non-specific binding was determined in the presence of 1 µM unlabeled N/OFQ. Total binding and non-specific binding were determined in duplicate for 12 concentrations of [^{3}H] N/OFQ within each assay. A Brandell multi-channel harvester was used to separate bound from free [^{3}H] N/OFQ by rapid filtration of the binding reactions through GF/B glass fiber filters that had been pretreated with 0.2% polyethyleneimine (PEI) for 60 min at 4° C. Filters were washed two times in the harvester with 2 ml of ice-cold 50 mM Tris-buffer pH 7.4. After washing the filter discs were punched out into scintillation fluid and incubated overnight at room temperature on a rotating shaker in 3.5 ml of scintillation fluid (Fisher) then counted on a β–counter for 1 min. Data were analyzed using Microsoft Excel™ and curves fit using Prism™ software.

cAMP Assays

HEK cells transiently expressing the indicated mNOR and hD$_1$-R were incubated in PBS containing 0.02% EDTA 2-4 min at 37°C and harvested by scraping and pipeting. Plates were washed with Krebs-Ringer (KRH) buffer (140 mM NaCl, 5 mM KCl, 2 mM

$CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 6 mM glucose and 25 mM Hepes/NaOH, pH 7.4) and cells pelleted by centrifugation at 800 rpm for 10 min. Cells were washed with KRH buffer and finally resuspended in KRH buffer. Cells were counted and approximately 10^6 cells were pre-incubated in the presence of various concentrations of N/OFQ in KRH buffer containing 100 μM 3-isobutyl-1-methylxanthine (IBMX) for 10 min at 37°C. cAMP accumulation was stimulated with 100 nM SKF-38393, a potent D_1 agonist, for 20 min at 37°C. The reaction was terminated by addition of equal volume of 0.5 mM sodium acetate buffer (pH 6.2) and boiling for 20 min. Samples were then centrifuged at 3000 rpm for 15 min to remove cellular material and supernatants stored at
-20°C until cAMP detection was carried out. Basal cAMP was measured in the absence of all drugs and maximal stimulation was measured in the presence of 100 nM SKF-38393 and the absence of N/OFQ. cAMP levels were determined using a [3H] cAMP radioimmunoassay kit (Diagnostic Products Corporation).

For the SKF-38393 dose response curve HEK cells transiently expressing the hD_1-R and the wild type mNOR were prepared as described above. Cells were incubated in the presence of various concentrations of SKF-38393 and in the absence and presence of simultaneously added 3 nM N/OFQ for 20 min at 37°C. The reaction was terminated and samples treated as described above.

To evaluate whether the mNORs exhibited any intrinsic activity, cells were transfected with the hD1-R alone or co-transfected with the hD1-R and each of the mNORs and cAMP accumulation assayed in the presence and absence of drugs. Cells were transfected as described above and empty pcDNA (Invitrogen) vector co-transfected with the hD1-R control such that total DNA transfected for each sample was constant. Cells were harvested as described and pre-incubated with 300 nM or 10 μM N/OFQ for 10 min at 37°C. Basal cAMP levels were determined in the absence of all drugs and cAMP accumulation was stimulated in all other samples with 100 nM SKF-38393 38393 for 20

min at 37°C. The reactions were terminated and cAMP measured as described. Data were analyzed and curves fitted using Prism™ software.

Statistical Analyses

Statistical significance was determined using Prism™ software by one-way analysis of variance (ANOVA) and the Kruskal-Wallis post test.

Results

Table 1 summarizes the saturation binding results obtained with the wild-type and mutant mNORs heterologously expressed in COS-7 cells. The mNOR R299A mutant appeared to possess approximately 5-fold higher affinity (K_d 24.3 ± 8.65 pM) for [^{3}H] N/OFQ than did the wild-type mNOR (K_d 120.8 ± 46.86 pM). Figure 1 illustrates a representative saturation curve of the wild-type mNOR and the R299A mutant receptor. Both mNOR D107A (Kd 1.017 ± 0.336 nM) and mNOR D107A/R299A (Kd 3.485 ± 2.047 nM) displayed greatly reduced affinity for [^{3}H] N/OFQ compared with the wild-type receptor. These results suggest that mutating R299 altered mNOR's conformation such that the ligand binding pocket is less stringent whereas the D107 residue plays an important role in ligand binding because mutation of this residue to alanine results in significantly reduced binding affinity.

In order evaluate the functional capacity of the wild-type and mutant mNOR's we detected N/OFQ mediated inhibition of cAMP accumulation in HEK cells transiently co-expressing hD1-R and each of the mNOR's. Instead of stimulating adenylyl cyclase with forskolin we stimulated cAMP production via activation of hD_1-R because our transient

Table 1

Results of Saturation Binding of [^{3}H] N/OFQ to mNORs expressed in Cos-7 Cell

Receptor	average K_d (pM)	SEM	n
mNOR	105.2	±46.86	6
mNOR R299A	24.3	±8.65	4
mNOR D107A	1017	±335.5	3
mNOR D107A/R299A	3485	±2047	3

Figure 1

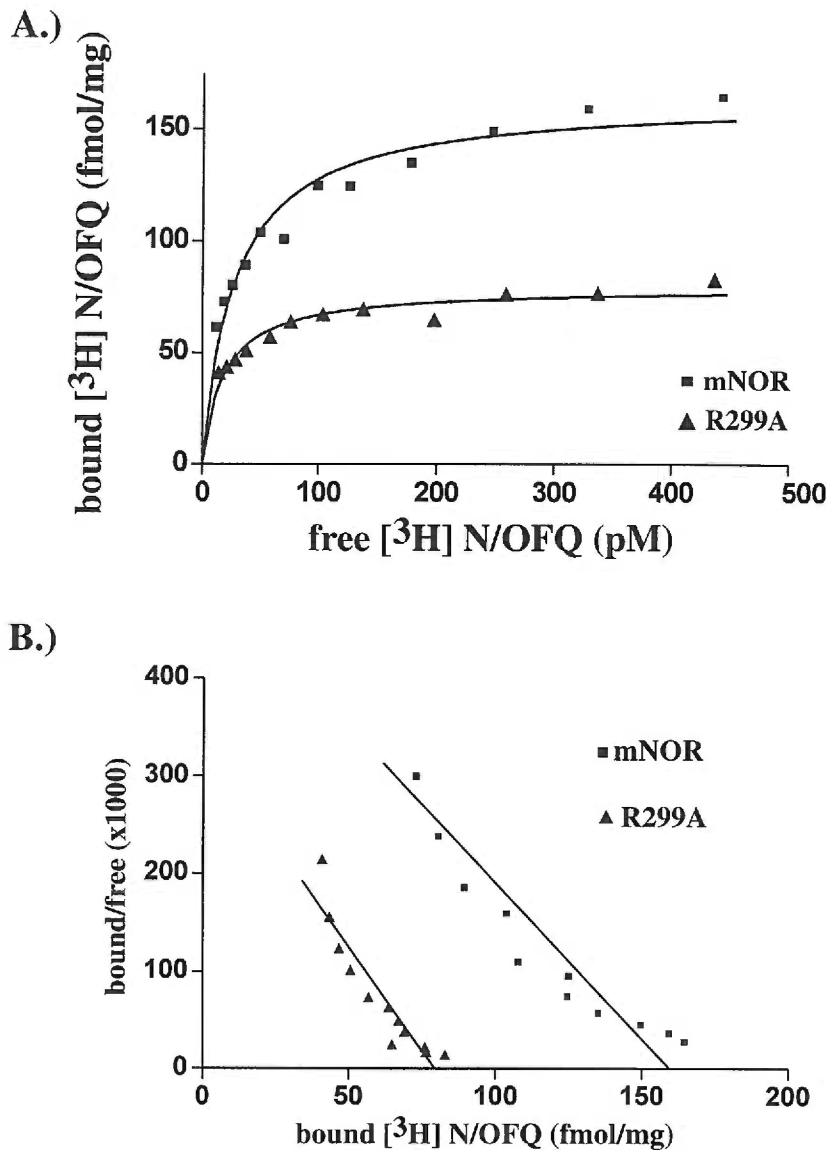

Figure 1. A representative saturation binding plot for COS-7 cell membranes expressing wild-type mNOR and mNOR R299A. Free [^{3}H] N/OFQ concentrations ranged from approximately 20 pM-500 pM. Curves represent specific binding determined by subtracting non-specific binding, in the presence of 1 μM unlabeled N/OFQ, from total binding. Kd values are 28.36 pM and 18.54 pM for mNOR and mNOR R299A, respectively. Bmax values are 163.70 fmol/mg and 79.27 fmol/mg for mNOR and mNOR R299A, respectively. B). The scatchard transformation of the binding curves.

transfection efficiency was only approximately 50%. A dose-response curve for the dopamine receptor-specific agonist, SKF-38393, was carried out to determine an appropriate concentration to use for the mNOR cAMP experiments. SKF-38393 (3 nM-3 μM) stimulated adenylyl cyclase activity in a dose dependent manner in HEK cells co-transfected with the hD_1-R and mNOR (Fig. 2). N/OFQ (3 nM) shifted this SKF-38393 dose-response curve to the right, resulting in an increased EC_{50} value of 35.5 nM compared with EC_{50} = 19.1 nM in the absence of N/OFQ. These results suggest that N/OFQ inhibited SKF-38393-mediated adenylyl cyclase activity via activation of mNOR and its coupled $G_{i/o}$ protein. In order to achieve robust N/OFQ mediated adenylyl cyclase inhibition, the lowest concentration of SKF-38393 that resulted in maximal adenylyl cyclase stimulation (100 nM) was utilized for N/OFQ dose-response cAMP experiments.

N/OFQ activation of the mutant mNORs and subsequent inhibition of SKF-38393 mediated stimulation of adenylyl cyclase appeared to be less potent compared with that of the wild-type mNOR (table 2). In spite of an increase in binding affinity, N/OFQ was about 10-fold less potent at inhibiting SKF-38393 stimulated adenylyl cyclase activation at mNOR R299A (EC_{50} = 25.98 nM) compared with the wild type receptor (EC_{50} = 2.004 nM). N/OFQ activation of the D107A mutant and the D107A/R299A double mutant. receptors yielded EC_{50} values in the micromolar range (39.43 μM and 558.6 μM, respectively) for the inhibition of SKF-38393-mediated stimulation of adenylyl cyclase. Although at N/OFQ concentrations that resulted in maximal inhibition for the wild-type receptor, D107A-containing mutant receptors exhibited undetectable adenylyl cyclase inhibition (Fig. 3).

In order to detect intrinsic activity of the mutagenized receptors, basal and SKF-38393 stimulated cAMP levels in cells co-expressing hD_1-R and each of the mNORs were compared with levels in cells expressing hD_1-R alone. We detected no difference in basal and stimulated cAMP levels in all cell types (Fig. 4). To evaluate the expression and functionality of mNORs in the co-transfected cells, SKF-38393 mediated stimulation of

Figure 2

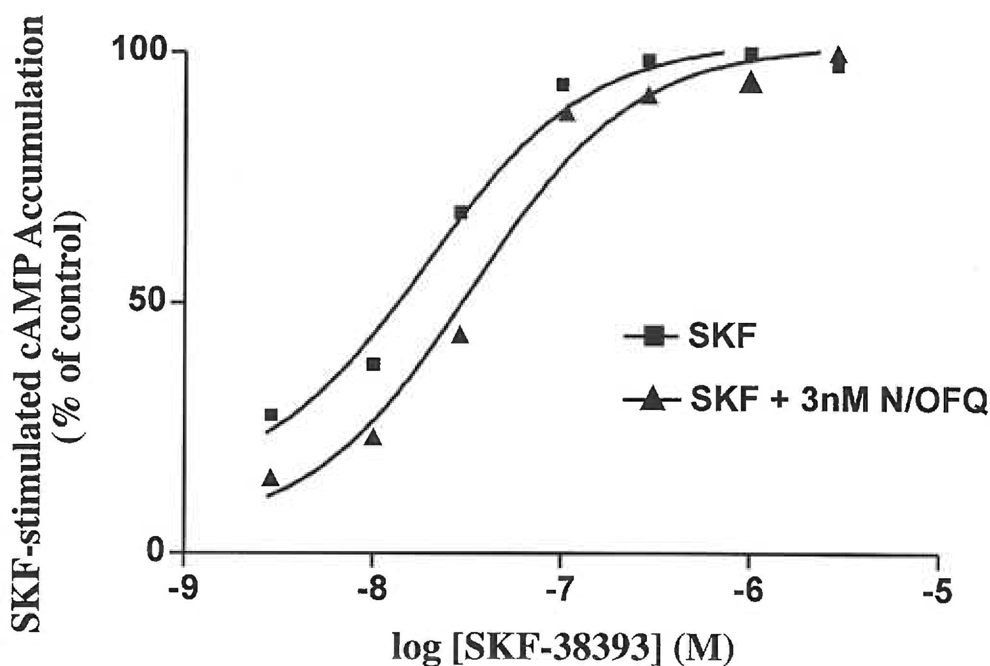

Figure 2. SKF-38393 dose-response curves. HEK cells transiently expressing the hD_1-R and mNOR were treated with SKF-38393 (3 nM-3 μM). cAMP accumulation was determined relative to maximum [^{3}H] cAMP bound in the absence of unlabeled cAMP ([^{3}H] cAMP Assay Kit, Diagnostic Products Corporation, Los Angeles, CA). Presence of 3 nM N/OFQ inhibited adenylyl cyclase, resulting in a shift to the right of the SKF-38393 dose-response curve. Percent stimulation was measured relative to unstimulated controls (in the absence of SKF-38393). EC_{50} values were 19.1 nM for SKF-38393 alone and 35.5 nM in the presence of N/OFQ.

Table 2

Results of N/OFQ-Mediated Inhibition of SKF-38393-Stimulated Adenylyl Cyclase in Transfected HEK Cells

Receptor	average EC_{50}	SEM	n
mNOR	2.004 nM	± 0.328	4
mNOR R299A	25.98 nM	±16.38	3
mNOR D107A	39.43 μM	± 18.26	3
mNOR D107A/R299A	558.6 μM	±515.7	3

adenylyl cyclase was inhibited in the presence of 10 μM and 300 nM N/OFQ (data not shown). Furthermore, N/OFQ had no effect on SKF-38393 mediated cAMP accumulation in cells transfected with the hD1-R construct alone (data not shown). Cells transfected with empty pcDNA vector failed to exhibit an SKF-38393 mediated increase in cAMP accumulation and showed no difference in basal cAMP levels compared with all other samples (data not shown).

Cells co-transfected with the hD1-R, the wild type mNOR and the GFP construct were counted to determine transfection efficiency. Approximately 47.8% of the cells counted (416/871) expressed the green fluorescent protein, suggesting a similar transfection efficiency for the receptors.

Discussion

The present study used site-directed mutagenesis to examine the activation mechanism of mNOR. Protein modeling suggested that the amino acid residues D107 and R299, located near the extracellular surface in TMD II and VII, respectively, face toward the interior of the receptor protein as it is inserted in the plasma membrane. It is reasonable to hypothesize that these oppositely charged residues form an interhelical salt bridge. The rhodopsin and α_{1b}-adrenergic receptors appear to contain such interhelical salt bridges, disruption of which appears to be sufficient for switching the receptor to the active conformation. Indeed, site-directed mutagenesis of residues involved in salt bridge formation in these receptors, thus disrupting the salt bridge linkage, results in constitutive activity of the receptors. Using site-directed mutagenesis we have mutated mNOR residues D107 and R299 to alanine to determine if activation of mNOR involves disruption of a putative salt bridge between these two residues.

Figure 3

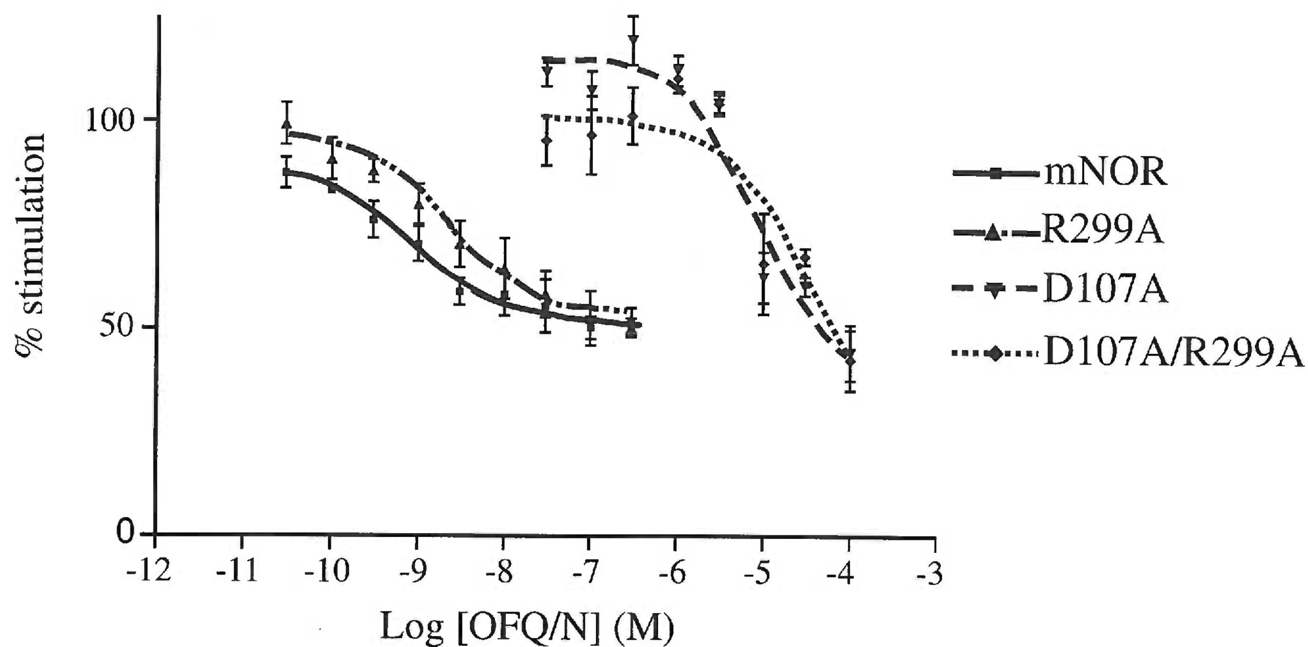

Figure 3. Dose-response curves for N/OFQ-mediated inhibition of SKF-38393 stimulated cAMP accumulation. HEK cells co-expressing hD1-R and the indicated mNOR were treated with 100 nM SKF-38393 a D_1-R agonist to activate adenylyl cyclase. N/OFQ (30 pM-100 μM) dose-dependently inhibited SKF-38393-mediated adenylyl cyclase activity. Percent stimulation was determined relative to fully stimulated SKF-mediated adenylyl cyclase activity in the absence of N/OFQ. Average EC_{50} values were 2.004 nM, 25.98 nM, 39.43 μM and 558.6 μM for mNOR, mNOR R299A, mNOR D107A and mNOR D107A/R299A, respectively.

Figure 4

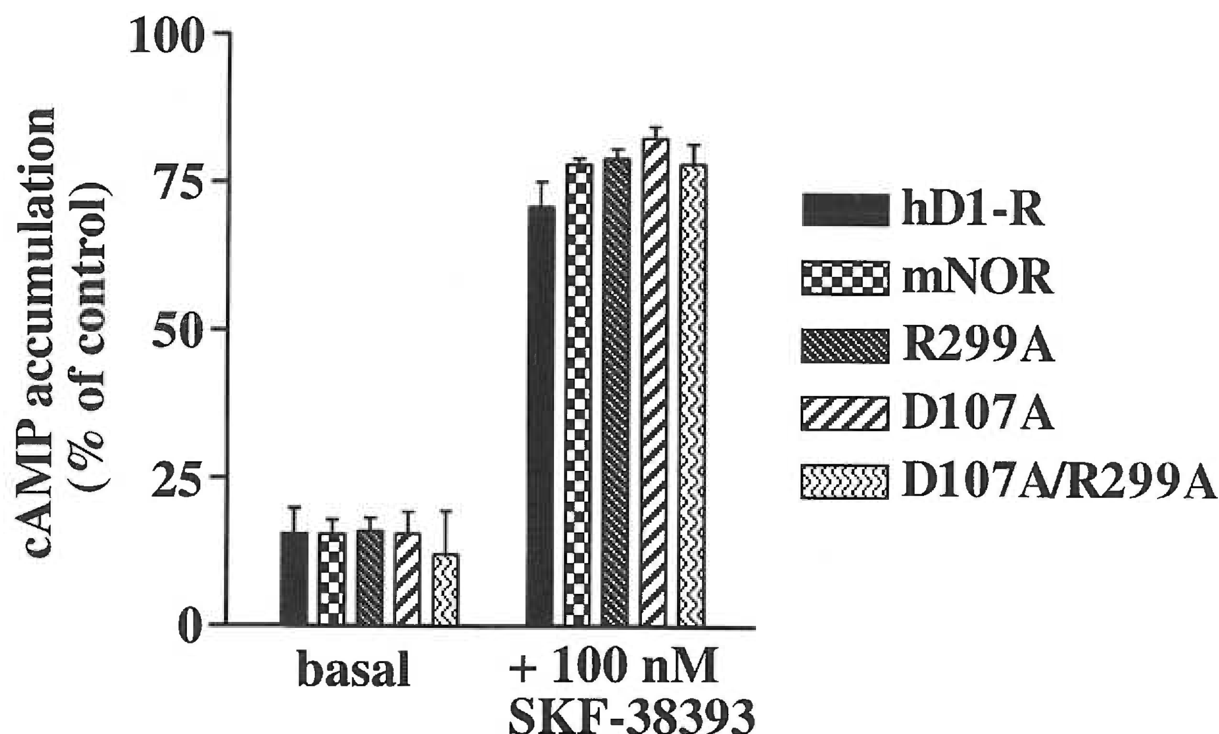

Figure 4. SKF-38393 mediated stimulation of cAMP accumulation. HEK cells were transfected with the hD1-R construct alone or co-transfected with the hD1-R and each of the mNOR constructs to detect any endogenous activity of the mNOR in the absence of N/OFQ. Basal adenylyl cyclase stimulated cAMP accumulation was determined in the absence of all drugs and adenylyl cyclase activity was stimulated in the presence of 100 nM SKF-38393. cAMP accumulation was determined relative to maximum [^{3}H] cAMP bound in the absence of unlabeled cAMP ([^{3}H] cAMP Assay Kit, Diagnostic Products Corporation, Los Angeles, CA). No significant difference ($p>0.05$) in adenylyl cyclase-mediated cAMP accumulation was determined under basal or stimulated conditions for the cell line expressing hD1-R only (n=3) compared with each of the co-transfected cell lines (n=3 each receptor type). This suggests that the mNORs possess no activity in the absence of N/OFQ. SKF-38393-mediated adenylyl cyclase stimulation was inhibited by 10 mM and 300 nM N/OFQ in co-transfected cell lines to confirm expression and functionality of mNORs (data not shown).

If mNOR is indeed constrained by a putative salt bridge then one might predict that disruption of the salt bridge would relax the stringency of the binding pocket and result in higher N/OFQ binding affinity. In the case of the R299A mutation this prediction holds, however, N/OFQ exhibits greatly reduced binding affinity for mNOR D107A and mNOR D107A/R299A compared with that of the wild type receptor. Of interest, these results are consistent with those observed for epinephrine binding to homologous α_{1b}-adrenergic receptor mutants (Porter, *et al.*, 1996). These results suggest that either, like the α_{1b}-adrenergic receptor, mutagenesis of the salt bridge forming residues results in a higher affinity binding state of mNOR but the D107 residue plays an additional role in high affinity ligand binding or that the residues do not form a salt bridge and the observed differences in binding affinity are a result of receptor-ligand charge interactions. Considering the former hypothesis, that mNOR residues D107 and R299 form a salt bridge that is involved in constraining the receptor in the inactive state, then one would expect that disruption of this putative salt bridge results in constitutive activity of mNOR, a result not observed here. The possibility that the observed differences in N/OFQ affinity for the mutant receptor are simply due to charge-charge interactions is a likely hypothesis.

The N/OFQ ligand contains many positively charged amino acid residues that might be repelled by the R299 residue in the receptor binding site. This residue may be important for stringency in ligand binding, mutation of which would be expected to reduce stringency of the binding site for positively charged ligands. Moreover, the D107 residue in the binding site would be predicted to attract binding of positively charged ligands. The mutant receptor binding results observed here are consistent with the hypothesis that receptor ligand binding is highly dependent on these charged residues. As would be predicted the mutation of the positively charged residue, arginine, to an uncharged residue results in increased N/OFQ binding affinity and, in contrast, mutation of the negatively charged aspartic acid to alanine markedly reduces N/OFQ binding affinity. It is interesting that the mNOR D107A/R299A double mutant results in a further reduction in N/OFQ binding

affinity compared with the NOR D107A mutant. One might predict that N/OFQ binding to the double mutant would yield some intermediate K_d value, between that obtained for N/OFQ binding to the R299A mutant and the D107A mutant. The observed result might be due to disruption of NOR structural integrity in the doubly mutated receptor. That N/OFQ binding affinity is much reduced in mutant receptors containing the D107A mutation compared with mNOR R299A or wild type NOR suggests that the D107 residue plays a more influential role than does the R299 residue in N/OFQ binding affinity. Furthermore, the R299A mutation does not compensate for loss of N/OFQ affinity due to the D107A mutation in the double mutant receptor.

It is interesting that the results of the saturation binding studies suggest a one-site model for N/OFQ binding to NOR. Since N/OFQ acts as an agonist at NOR one might expect to observe a two-site binding model. That is, agonists binding to a given GPCR is predicted to activate the receptor and uncouple the G protein, thus switching the receptor to the active conformation. Since the receptor is uncoupled in the active conformation, the activated GPCR possesses reduced binding affinity for agonist. Therefore, in the case of agonist binding one expects to observe two affinity states of the receptors, coupled and uncoupled. Furthermore, one expects to obtain two K_d values, one that represents the high and low affinity states of the receptor. The data plotted as a scatchard transformation should represent two lines of steep and shallow slope for each of the affinity states (a two-site fit) as opposed to one line in the case of a single affinity state of the receptor (a one-site fit). The results obtained for N/OFQ binding to each of the NORs tested here suggest a one-site model. One reasonable explanation for these results is that relatively low concentrations of the N/OFQ agonists are used in these studies which may only bind to NORs in the high affinity state (coupled). Binding affinity (K_d) values reported here are comparable with high affinity values reported in the literature, suggesting that this may indeed be the case. One might expect that if a wider range and higher concentrations of

N/OFQ were tested at these NORs, the receptors in the lower affinity state would also be bound and a two-site binding model might be observed.

The observed N/OFQ binding affinity of the mutant NORs may be in at least in part due to conformational changes in the ligand binding pocket as a result of alanine substitution for larger native amino acids. Alanine substitution for either arginine or aspartic acid not only removes the respective charges of these amino acids but also introduces a much smaller side group. The side group of alanine is simply a methyl group compared with the rather large and complex side chains of arginine or aspartic acid. In mutagenesis studies amino acids are commonly substituted with alanine due to its aliphatic nature and small side chain. One caveat of substituting alanine for arginine or aspartic acid is the difference in the size and structure of the amino acid side chains. One cannot rule out the possibility that the observed difference in the wild-type NOR and the mutant NORs tested here is simply due to a conformational change in NOR caused by the size and structure of the alanine side chain and not due to removal of the positive or negative charge. If this were indeed the case, one can not make any predictions about the interactions of D107 and R299 with other receptor amino acids or with N/OFQ amino acids. One possible way to address this issue is to replace D107 or R299 with amino acids that are more similar in size and strucure without introducing reactive side groups.

The hypothesis that mNOR residues D107 and R299 are involved in N/OFQ-mNOR charge interactions is supported by N/OFQ mutagenesis studies. Alanine scanning studies of N/OFQ indicate that the arginine residue at position 8 (R8) is essential for high affinity binding and functional potency (Dooley and Houghten; Reinscheid *et al.*, 1996). Furthermore, replacement of N/OFQ R8 with D-arginine has less profound consequences on receptor binding and activation than does alanine substitution (Reinscheid *et al.*, 1996). This suggests that a positive charge at this position and, to a lesser extent, its orientation are important for receptor binding and activation (Reinscheid *et al.*, 1996). The N/OFQ R8 residue may interact with the mNOR D107 side chain to confer high affinity binding. In

contrast, the NOR R299 residue may repel N/OFQ R8 and therefore the mutation R299A would be expected to result in higher affinity N/OFQ binding.

An interesting observation in these mNOR mutagenesis studies is the seemingly paradoxical result that N/OFQ possessed increased binding affinity yet reduced functional potency at mNOR R299A compared with the wild type mNOR. This result suggests that receptor binding and activation may be mediated via different mechanisms and that different domains of the receptor or peptide may be responsible for each of these functions. The "message-address" hypothesis for opioid peptides suggests that binding and activation of opioid receptors are mediated via different domains of the peptide ligand. Opioid receptor activation is believed to primarily be mediated by the "message" domain or N-terminal portion of opioid peptides whereas the C-terminal portion of opioid peptides is thought to constitute the "address" domain, the portion of the ligand responsible for receptor selectivity (Chavkin and Goldstein, 1981). Considering the relatively positively charged nature of the C-terminal or "address" portion of N/OFQ, the R299A mutation would be expected to confer a better binding site for N/OFQ than does the wild type mNOR. The observed reduction in "message" efficacy or mNOR R299A activation may be due to altered conformation of N/OFQ in the higher affinity binding site. The opioid 'message-address" hypothesis is one theory that may explain the apparent disparity in the results observed for N/OFQ binding affinity and receptor activation of the mNOR R299A mutant. The reduced potency of N/OFQ at mNOR D107A and mNOR D107A/R299A mutants may simply be reflective of the observed reduction in N/OFQ binding affinity.

In a recent report, Mollereau et al. observed similar counterintuitive results of increased opioid binding affinity yet reduced receptor activity for a mutagenized NOR (Mollereau *et al.*, 1996b). The NOR residue glutamine 280 located in TMD VI was replaced with histidine (Q280H). The purpose of this mutation was to create an opioid binding pocket since the His is conserved among all three classical opioid receptors (Mollereau *et al.*, 1996b). This NOR mutation has no effect on N/OFQ binding affinity or

functional potency in the inhibition of adenylyl cyclase (Mollereau *et al.*, 1996b). Interestingly, the opioid agonist lofentanil exhibits increased binding affinity and reduced functional activity for NOR Q280H (Mollereau *et al.*, 1996b). These results are similar to those observed for N/OFQ acting at mNOR R299A. However, these two NOR mutagenesis studies are not necessarily comparable because lofentanil is not the endogenous ligand for NOR. Furthermore, the NOR Q280H mutation markedly affects both the functional potency and efficacy of lofentanil at NOR, whereas N/OFQ exhibited only a notable reduction in potency at NOR R299A compared to the wild type NOR.

The observation that the mutant mNORs do not appear to be constitutively active refutes the hypothesis of the studies presented here. However, inhibition of intracellular cAMP accumulation may not be the most sensitive test of receptor activity. Ability to activate G protein coupled inwardly rectifying potassium channels (GIRKs) is one alternative method for examining the putative constitutive activity of the mutant mNORs. Measuring the activation of a second messenger pathway, such as GIRKs may be more accurate than measuring the inhibition of a stimulated pathway, such as the inhibition of adenylyl cyclase. Furthermore, inhibition of adenylyl cyclase activity via activation of a $G_{i/o}$ coupled receptor appears to be mediated by the $\beta\gamma$ subunit of the G protein, whereas the activation of GIRKs is mediated directly by the α subunit. In a cell culture system one can not predict the availability of G protein subunits, therefore more than one test of mNOR activity may be useful to draw the most accurate conclusions regarding the activity of the mutant mNORs.

The present studies suggest that activation of the mNOR is not mediated via disruption of an interhelical salt bridge linkage between residues D107 and R299. Therefore, the mechanism of mNOR activation likely differs from that of the rhodopsin and α_{1b}-adrenergic receptors. That a single amino acid substitution, R299A, results in a four-fold increase in N/OFQ binding affinity is an interesting observation. The fact that the observed N/OFQ binding affinity and activation of this mutant receptor appeared

paradoxical makes this NOR amino acid more intriguing. Mutagenesis of this residue to either a positively charged or negatively charged residue can provide support for the importance of charge interactions between mNOR and N/OFQ at this site and may lead to a better understanding of the mechanisms responsible for N/OFQ binding and activation of mNOR.

Discussion and Conclusions

N/OFQ RIA: a tool for studying peptide processing and regulation in stress and circadian rhythms

The RIA is a useful technique for detecting N/OFQ immunoreactivity in tissue or cell culture samples. The finding that N/OFQ$^{1-17}$ and preproN/OFQ (ppN/OFQ) mRNA are abundant in the hypothalamus suggests that ppN/OFQ is translated and the precursor protein cleaved to release bioactive peptides within the hypothalamus. The N/OFQ RIA detects N/OFQ$^{1-17}$-containing proteins including the N/OFQ precursor and partially processed intermediate proteins. That the N/OFQ$^{1-17}$ peptide is the primary N/OFQ$^{1-17}$-containing peptide detected in the hypothalamus and NOR expression is dense in several hypothalamic nuclei (Neal *et* al., 1999) suggests that N/OFQ$^{1-17}$ is involved in behaviors mediated by the hypothalamus. Given these data we hypothesized that N/OFQ$^{1-17}$ plays a role in stress.

The hypothalamic-pituitary-adrenal (HPA) axis is activated in response to stressful stimuli. Corticotrophin releasing hormone (CRH), secreted from the hypothalamus to the pituitary via the hypophyseal portal vein, stimulates pituitary biosynthesis and secretion of adrenocorticotrophic hormone (ACTH). ACTH stimulates the adrenal glands to synthesize and release adrenocortical hormones including glucocorticoids. The HPA axis is subject to feed-back inhibition at the hypothalamus and pituitary mediated by circulating glucocorticoids. The opioid system has been implicated in modulation of the HPA axis. Indeed, acute stress results in opioid mediated stress induced analgesia (SIA) and is accompanied by an increase in endogenous opioids in the brain (Akil, *et al.*, 1984). However, chronic stress leads to an adaptation in animals such that further stress no longer produces analgesia (Akil *et al.*, 1984).

Mogil *et al.* have shown that N/OFQ injected i.c.v. dose-dependently reverses acute opioid-mediated and naloxone-sensitive stress induced analgesia (SIA) (Mogil *et al.*, 1996). Furthermore, N/OFQ not only reverses endogenous opioid mediated analgesia

supraspinally but also dose-dependently reverses morphine-induced analgesia, suggesting that N/OFQ can act as a functional anti-opioid peptide (Mogil *et al.*, 1996). These data suggest that exposure to stressful stimuli may lead to an increase in N/OFQ peptide as a consequence of increased endogenous opioid peptides and that the anti-opioid properties of N/OFQ may be involved in adaptation during chronic stress. In our studies, we found that hypothalamic content of N/OFQ was reduced in mice that had been subjected to chronic restraint stress. This may be due to hypersecretion of N/OFQ or to an adaptation of the mice during chronically stressful conditions.

Lending further support to the idea that N/OFQ plays a role in adaptation to chronic stress, N/OFQ knock-out mice fail to adapt to stress, suggesting that N/OFQ could be involved in development of coping strategies in response to repeated stress (Köster *et al.*, 1999). This failure to adapt to repeated stress is accompanied by sustained induction of SIA and increased circulating glucocorticoids in these transgenic animals (Köster *et al.*, 1999). The N/OFQ knock-out mice exhibit an increased susceptibility to stress and display anxiety-like behavior in novel or threatening environments (Köster *et al.*, 1999). The inability of N/OFQ knock-out mice to adapt to chronic activation of the HPA axis, during repeated stress, is consistent with the idea that N/OFQ functions as an anxiolytic (Jenck, *et al.*, 1997). The results of these N/OFQ knock-out studies are confounded by the fact that the entire ppN/OFQ gene is disrupted in these mice. Therefore, not only do these transgenic animals lack N/OFQ but they also fail to express the other putative bioactive peptides contained within the N/OFQ precursor protein. Consequently, the failure of these transgenic animals to adapt to stress can not necessarily be attributed to lack of N/OFQ but may be due to other ppN/OFQ-derived peptides. In our studies, we used a model of chronic HPA axis activity, transgenic mice that over-produce corticotrophin-releasing hormone (CRH). The increase in hypothalamic N/OFQ observed in these animals may be due to increased biosynthesis of N/OFQ in an attempt to adapt to the chronic HPA axis activity. Since CRH presumably acts upstream of N/OFQ in activation of the HPA axis,

N/OFQ is unable to inhibit the CRH mediated HPA activity despite elevated peptide levels in these animals.

Both repeated stress (Takahashi *et al.*, 1988, 1989) and administration of glucocorticoids (Takahashi *et al.*, 1989) suppress the development of morphine tolerance in mice. Morphine tolerance and tolerance to opioid-mediated SIA are thought to be mediated via similar mechanisms (Köster *et al.*, 1999). Furthermore, N/OFQ knock-out mice exhibit elevated levels of plasma corticosterone and fail to adapt to opioid-mediated SIA during chronic stress states (Köster *et al.*, 1999). In our studies, chronic restraint stress or the administration of dexamethasone, a synthetic glucocorticoid, resulted in a reduction in hypothalamic N/OFQ content. Collectively, these data imply that a function of N/OFQ in the stress response is to functionally antagonize the effects of opioid-mediated SIA. Since the HPA response and the opioid system are intimately connected it is difficult to determine whether the primary function of N/OFQ in the stress response is that of an anxiolytic or an anti-opioid.

The results of in situ hybridization and N/OFQ RIAs performed on brain regions other than the hypothalamus suggest that the peptide may be involved in numerous physiological functions beside stress. For example, the detection of N/OFQ in the suprachiasmatic nucleus (SCN) suggests that the peptide may play a role in modulating circadian rhythms, the daily fluctuations in behavioral and physiological processes. The suprachiasmatic nucleus (SCN) is thought to be the primary regulator of the circadian cycle, receiving input directly from the retina, transmitted via the neurotransmitter glutamate (De Vries *et al.*, 1993; Meijer and Rietveld, 1989; Morin, 1994; Moore *et al.*, 1995). In situ hybridization and RIA indicated the presence of nociceptin/orphanin FQ receptor (NOR) mRNA and N/OFQ peptide, respectively, in the SCN. This finding suggests that the N/OFQ system may play a role in the establishment and/or maintenance of circadian rhythms.

The opioid peptide enkephalin has also been implicated in the regulation of circadian rhythmicity in the SCN (Cutler, *et al.*, 1999). Although in vitro basal and NMDA-activated firing rates of SCN neurons were unaltered in the presence of Met- or Leu-enkephalin or morphine (Cutler *et al.*, 1999), a rebound excitatory response did follow their washout. Naloxone administration following morphine treatment resulted in a similar excitatory response. This phenomenon was referred to as "withdrawal activation", and suggests that withdrawal from opiate use may affect the firing of SCN neurons and ultimately circadian function (Cutler, *et al.*, 1999). We found that N/OFQ activates outward K^+ currents in SCN neurons, an action that hyperpolarizes the membrane potential and renders the neurons less excitable. In contrast to the enkephalins, N/OFQ potently inhibits the activity of SCN neurons, underscoring the difference between N/OFQ and the classical opioid peptides. If N/OFQ is found to have anti-opioid activity in the SCN, one might expect that administration of the peptide might have similar overall effects as naloxone administration. Perhaps paradoxically, the effects on SCN neuronal excitation are quite the opposite. However, it can not be overlooked that the naloxone-mediated response was observed following administration of morphine.

Detection of N/OFQ by RIA in various tissues has lead to hypotheses about possible functional mechanisms for the peptide *in vivo*. Since ppN/OFQ message was undetectable in the SCN by in situ hybridization, the RIA proved to be a valuable method for detecting N/OFQ immunoreactivity in the SCN, supporting the hypothesis that N/OFQ can modulate activity of SCN neurons and affect circadian rhythms. Furthermore, since ppN/OFQ message was not detected in SCN neurons, N/OFQ peptide must be synthesized in neurons in other brain nuclei that project to the SCN or the mRNA is expressed at very low copy number. This suggests that N/OFQ activity in SCN neurons may be modulated via other nuclei and neuronal pathways.

Integrity of N/OFQ and putative function of degradation products

Wide variability in receptor binding affinity suggested that N/OFQ may be subject to degradation events during the binding experiment, affecting observed Kd values. In an effort to establish a reliable binding assay, we examined the integrity of the [^{3}H] N/OFQ peptide during storage and in various incubation conditions used during binding. The integrity of the [^{3}H] N/OFQ peptide was analyzed by reversed-phase high performance liquid chromatography (RP-HPLC).

Tritiated N/OFQ$^{1-17}$ obtained from three different commercial sources was analyzed for stability in storage and in binding assay incubation conditions. The peptide received from two of the sources was labeled at amino acid positions phenylalanine1 and 4 (F1,4), and the other source supplied a peptide labeled at position leucine14 (L14). Of interest, the position of the tritium label appeared to influence peptide stability. Indeed, the F1,4 labeled peptides were unstable during storage, and reliable binding results were not obtained with these peptides. In contrast, the L14-[^{3}H] N/OFQ remained intact during storage, and routinely yielded reproducible binding results. These findings suggested that the F1,4 labeled peptide may be subject to radiolysis. The process of labeling may account for the differences observed in intrinsic peptide stability. Alternatively, the position of the label may affect the sensitivity of the peptide to radiolysis.

In spite of the intrinsic stability of the L14-[^{3}H] N/OFQ peptide, we found that the peptide degrades during the binding assay. Given the presence of two putative dibasic amino acid cleavage sites (RK) within N/OFQ$^{1-17}$ peptide, it is possible that the peptide is processed by endopeptidases at these sites. Several investigators have examined the putative function of smaller peptides that might result from N/OFQ cleavage events (Dooley and Houghten, 1996; Guerrini *et al.*, 1997; Sandin *et al.*, 1999; Suder *et al.*, 1999). N/OFQ$^{1-13}$ binds NOR with high affinity, comparable to that of the full-length N/OFQ$^{1-17}$ peptide (Dooley and Houghten, 1996; Butour *et al.*, 1997; Guerrini *et al.*, 1997). Furthermore,

peptide fragments shorter than N/OFQ$^{1-13}$ (i.e. N/OFQ$^{1-12}$, N/OFQ$^{1-11}$, etc.) also bind to NOR, albeit with significantly reduced affinity compared with N/OFQ$^{1-17}$ (Dooley and Houghten, 1996; Butour *et al.*, 1997; Guerrini *et al.*, 1997; Rossi *et al.*, 1997). Considering that these peptides possess binding affinity for NOR but no longer contain the L14, the amino acid position of the tritium label, they may compete with the [3H] L14 labeled N/OFQ for NOR sites and therefore affect the observed binding affinity among different experiments. This putative binding to NOR of unlabeled N/OFQ fragments may account for some of the variability in binding affinities reported in the literature.

Binding to NOR of shorter N/OFQ-derived peptides generated by cleavage or degradation of N/OFQ suggests that these peptides may play a functional role distinct from that of the 1-17 amino acid peptide. The putative cleavage products N/OFQ$^{1-7}$ and N/OFQ$^{1-11}$ have been reported to mediate analgesia in CD-1 mice when injected intracerebroventricularly (i.c.v.) (Rossi *et al.*, 1997; Mathis, *et al.*, 1998). The N/OFQ$^{1-17}$ metabolite N/OFQ$^{1-6}$ is abundant in the spinal cord of Wistar rats (Suder *et al.*, 1999). The N/OFQ$^{1-6}$ cleavage product when injected i.c.v. in rats elicits a bi-phasic behavioral response, inducing analgesia up to 10 min after injection followed by hyperalgesia at 30 min post-injection (Suder *et al.*, 1999). Interestingly, this N/OFQ$^{1-6}$-mediated analgesia and hyperalgesia are reversed by the opioid receptor antagonist naloxone and by NMDA receptor antagonists, respectively (Suder *et al.*, 1999). Sandin et al. (1999) demonstrated that N/OFQ$^{1-13}$ is a major metabolite when N/OFQ$^{1-17}$ is injected into the rat hippocampus. In contrast with N/OFQ$^{1-17}$, the N/OFQ$^{1-13}$ fragment injected into the rat hippocampus fails to modulate spatial learning in the Morris water maze (Sandin *et al.*, 1999). These results suggest that despite high affinity binding and potent activation of NOR, N/OFQ$^{1-13}$ may mediate different behavioral effects *in vivo* compared with N/OFQ$^{1-17}$. Since N/OFQ$^{1-17}$ can impair spatial learning when injected into the hippocampus (Sandin *et al.*, 1997), cleavage of N/OFQ$^{1-17}$ by hippocampal enzymes may serve to protect the organism from this behavioral effect mediated by the peptide. These *in vivo* studies suggest that N/OFQ$^{1-17}$-derived

peptides do in fact possess biological activity and produce behavioral effects that differ from those of N/OFQ^{1-17}. Additionally, these N/OFQ-derived peptides may act through NOR, receptors other than NOR or through some form of heteromeric receptors.

Recent studies by Mathis et al. (1997) provide evidence for NOR receptor heterogeneity in mouse brain thus supporting the hypothesis that putative N/OFQ^{1-17}-derived peptides or N/OFQ^{1-17} may act through receptors other than NOR. Receptor heterogeneity may account for some of the differences in behavioral effects induced by N/OFQ^{1-17} compared with its putative cleavage products. NOR heterogeneity may also account for some of the controversy over behavioral effects mediated by N/OFQ^{1-17}. In mice, N/OFQ^{1-17} has been shown to both increase (Florin *et al.*, 1996) and decrease (Reinscheid *et al.*, 1995) locomotion when injected i.c.v. One possible explanation for these opposing results is the dose of N/OFQ^{1-17} administered. Higher doses of peptide decrease locomotion, whereas increased locomotion results from lower doses. N/OFQ^{1-17} cleavage is one possible explanation for the observed differential behavioral effects in response to the amount of peptide administered. At low doses, a lesser amount of N/OFQ may remain uncleaved to mediate N/OFQ behavioral responses and therefore different behavioral responses mediated by N/OFQ cleavage products acting at different receptors are observed. Disparate effects on feeding behavior are also attributed to N/OFQ^{1-17} depending on the physiological state of the animals (Pomonis *et al.*, 1996; Candeletti *et al.*, 1998). Injection of N/OFQ^{1-17} i.c.v. can reduce feeding in fasted rats (Candeletti *et al.*, 1998) and in sated rats stimulates feeding (Pomonis *et al.*, 1996). The physiological condition of the animal may also influence the expression of neurotransmitter receptors and/or cleavage enzymes, whose activity can generate N/OFQ^{1-17}-derived peptides.

Evidence in support of the hypothesis that endopeptidase cleavage of N/OFQ^{1-17} is biologically relevant includes *in vivo* studies of rat hippocampus in which N/OFQ^{1-17} appears to be metabolized initially to N/OFQ^{1-13} and N/OFQ^{14-17}. The N/OFQ^{1-13} peptide is subsequently cleaved to N/OFQ^{1-9} and N/OFQ^{10-13} (Sandin *et al.*, 1999). In the spinal cord

of rats, the processing of N/OFQ$^{1-17}$ appears to be mediated by a serine endopeptidase that releases N/OFQ$^{1-11}$ which is subsequently cleaved to N/OFQ$^{1-6}$ (Suder *et al.*, 1999). N/OFQ$^{1-17}$ is also subject to processing *in vitro*. The pattern of N/OFQ$^{1-17}$ cleavage is similar in SH-SY5Y human neuroblastoma, human lung carcinoma cell lines and in primary cultured cells derived from rat cortex (Vlaskovska *et al.*, 1999). The cleavage of N/OFQ$^{1-17}$ appears to be mediated by a metallosensitive endopeptidase with the major products being N/OFQ$^{1-9}$ and N/OFQ$^{1-13}$ (Vlaskovska *et al.*, 1999). Interestingly, chronic morphine treatment of these cultured cells potentiates the enzymatic activity and results in the accumulation of other cleavage products, N/OFQ$^{1-12}$, N/OFQ$^{1-6}$ and N/OFQ$^{1-5}$ (Vlaskovska *et al.*, 1999).

The identification of other endopeptidases that can cleave N/OFQ have been pursued by Montiel et al. who demonstrated the ability of aminopeptidase and endopeptidase 24.15 to recognize and cleave N/OFQ$^{1-17}$ in mouse cortical brain slices (Montiel *et al.*, 1997). In a subsequent experiment, Noble and Roques examined the activity of these peptidases in N/OFQ$^{1-17}$ processing *in vivo* by co-injection of N/OFQ$^{1-17}$ and specific protease inhibitors (Noble and Roques, 1997). The injection of N/OFQ$^{1-17}$ i.c.v. in mice impairs motor activity in both the open field and spontaneous motor activity (Noble and Roques, 1997). Interestingly, co-administration of N/OFQ$^{1-17}$ and protease inhibitors specific for aminopeptidase and endopeptidase 24.15 potentiated this impairment of motor activity (Noble and Roques, 1997). These observations suggest that the protease inhibitors result in a higher effective concentration of intact N/OFQ$^{1-17}$ which can then result in a more pronounced behavioral response. These results also suggest that these proteases may play a role in abolishing the effects of neuropeptides *in vivo*.

When taken together, the data suggest that N/OFQ$^{1-17}$ is sensitive to enzymatic cleavage both *in vitro* and *in vivo*. Our analysis of [^{3}H]N/OFQ degradation underscores the importance of standardizing labeling, storage and handling of the peptide in order to ensure its integrity and to obtain consistent experimental results. Indeed, integrity of the peptide

appears to influence observed behavioral effects. The *in vivo* studies suggest that enzymatic cleavage may be an important component of N/OFQ$^{1-17}$ regulation. It is also an intriguing possibility that N/OFQ$^{1-17}$ -derived cleavage products possess some additional biological activity of their own, and that the activity of these putative peptides may be mediated via receptors distinct from NOR.

Examining mechanisms of receptor-ligand interactions

The mechanism of G protein-coupled receptor (GPCR)-ligand interactions has long been of interest. Current opinion holds that distinct domain(s) in both the receptor and its ligand are responsible for high affinity binding and coupling to second messenger pathways attributed to these proteins. The ligand domain(s) that mediate high affinity binding, may not be the same as the domain(s) essential for the ability of the ligand to activate its receptor. This is reflected in of the "message-address" hypothesis developed for the opioid peptides. This hypothesis states that opioid peptides contain a message domain that confers activity and an address domain that is responsible for receptor selectivity and increased potency (Chavkin and Goldstein, 1981). Much of the experimental evidence suggests that NOR-N/OFQ may not follow this simple opioid model of the "message-address" mechanism for binding and receptor activation (Butour *et al.*, 1997; Lapalu *et al.*, 1997; Reinscheid *et al.*, 1996). However, not all studies completely refute the "message-address" hypothesis for N/OFQ binding (Dooley and Houghten, 1996; Guerrini *et al.*, 1997).

Many investigators have tried to determine which amino acids or domains are essential for high affinity binding of N/OFQ and functional efficacy (Table 1). Alanine scanning mutagenesis of N/OFQ, in which each amino acid in the peptide is replaced with an alanine (A) residue, revealed that residues 1,2,4,5 and 8 are essential for high affinity binding of N/OFQ to NOR (Dooley and Houghten, 1996; Reinscheid *et al.*, 1996).

Interestingly, the N-terminus of N/OFQ (F-G-G-F) differs from the canonical N-terminal sequence of opioid peptides (Y-G-G-F) by a single hydroxyl group, and this important distinction was thought to be sufficient to confer NOR selectivity. Surprisingly however, substitution of N/OFQ F1 with Y had no effect on binding affinity or receptor activation (Reinscheid *et al.*, 1996). When A is substituted for F1, the resulting peptide displays greatly reduced affinity for NOR and virtually no potency (Dooley and Houghten, 1996; Reinscheid *et al.*, 1996) suggesting that the aromatic side group of the residue at position one is essential for high affinity binding and functional coupling. In contrast, substitution of the F4 residue with Y significantly reduced functional potency of the peptide yet had little effect on binding affinity (Reinscheid *et al.*, 1996). Therefore, F4 appears to be involved in receptor activation, since substitution for A (Reinscheid *et al.*, 1996) or L (Guerrini *et al.*, 1997) leads to inactivity.

The substitution of A for R8 resulted in a reduction of N/OFQ binding affinity and abolished receptor function (Dooley and Houghten, 1996; Reinscheid *et al.*, 1996). In contrast, D-amino acid substitution of R8 had less dramatic effects on receptor binding and functional coupling, implying that a positively charged residue at position eight is more essential than the exact orientation of the charge (Reinscheid *et al.*, 1996). Guerrini et al. confirmed the importance of R8 for receptor activation and went on to demonstrate that all N/OFQ cationic residues (R8,12 and K9,13) are essential for receptor activation (Guerrini *et al.*, 1997).

Truncation mutagenesis of N/OFQ is another approach that has been explored in an effort to determine the N/OFQ domains involved in NOR binding and activation. Serial truncation of C-terminal residues revealed that the shortest N-terminal N/OFQ fragment that retains high affinity NOR binding is N/OFQ$^{1-13}$ (Dooley and Houghten, 1996). Furthermore, Guerrini et al. demonstrated that N/OFQ$^{1-13}$ inhibits electrically evoked twitches of mouse vas deferens as potently as does N/OFQ$^{1-17}$ (Guerrini *et al.*, 1997). However, these results were obtained with N/OFQ$^{1-13}$ which has been amidated C-terminally

(N/OFQ^{1-13}-NH_2) which is essential to protect N/OFQ^{1-13} from degradation by proteases (Dooley and Houghten, 1996; Guerrini *et al.*, 1997). N-terminally truncated N/OFQ peptides, N/OFQ^{2-17}, N/OFQ^{6-17} and N/OFQ^{12-17}, retain modest NOR binding affinity and activation (Butour *et al.*, 1997). Taken together, these data argue against the idea of opioid peptide-like "message-address" domains for N/OFQ, since N- or C-terminal truncations result in peptides that retain ability to both bind and activate NOR. It is intriguing, however, that all N- or C-terminally truncated peptides that retain function must contain the positively charged residues R12 and K13, underscoring their involvement in receptor-ligand interactions.

To date, several studies have been carried out to determine which N/OFQ amino acid residues are critical for NOR binding and activation. However few investigators have examined which NOR amino acid residues and domains are involved in N/OFQ interaction. Consequently, we created NOR mutants using site-directed mutagenesis in an effort to identify NOR residues that are involved in N/OFQ binding and subsequent NOR activation. Our studies of NOR recapitulate the importance of positively charged N/OFQ amino acid residues for high affinity binding and receptor activation. Mutagenesis of NOR residue D107, located near the extracellular face of TMD III, to an A led to a great reduction of N/OFQ binding affinity and functional potency at the receptor. These results, together with the N/OFQ mutagenesis studies, suggest that high affinity N/OFQ binding and potent NOR activation are mediated to a significant degree by ligand-receptor charge interactions. Moreover, mutagenesis of the NOR residue R299 to A, in TMD VII, resulted in higher N/OFQ binding affinity. This observation implies that the positively charged R residue in this position of NOR may electrostatically repel the positively charged N/OFQ peptide and that removal of the NOR positive charge, R299, results in higher affinity of N/OFQ for NOR.

The opioid peptide "message-address" hypothesis has been useful in our efforts to explain some of the results obtained in our mNOR mutagenesis studies. When the "message-address" hypothesis is applied to the dynorphin A peptide, an endogenous kappa opioid receptor (KOR) agonist, the first five amino acid residues are considered to constitute the message, which confers opioid activity. The C-terminal residues of dynorphin constitute the address that is thought to be involved in selective binding and functional potency at KOR (Chavkin and Goldstein, 1981). In our NOR site-directed mutagenesis studies we found that the R299A receptor mutation resulted in higher N/OFQ binding affinity but reduced efficacy, which suggests that binding and activation of NOR are not necessarily interconnected mechanisms. Since the "message-address" hypothesis implies that different domains of the peptide ligand are involved in receptor binding and activation, it is an attractive explanation for these seemingly counter-intuitive results obtained with the NOR R299A. Considering this hypothesis, the R299 residue appears to be more important for functional potency than for selective binding, since substitution of this residue with A yielded reduced receptor activity. When the NOR R299A mutation was evaluated, it did not display reduced N/OFQ binding affinity. In fact, the R299A mutation resulted in an increased binding affinity. This observation suggests that R299 is not important in message recognition. However, mutating this residue to A altered the conformation of NOR such that higher affinity binding is achieved. Alternately, R299 may interact with N/OFQ's "address" domain in such a way that removal of the positively charged R residue results in a receptor with higher affinity but reduced efficacy due to a conformational change of the message recognition site. At any rate, the presumably paradoxical results observed for the effects of the R299A mutation on binding affinity and functionality are intriguing, suggesting that N/OFQ binding and activation of NOR are not necessarily parallel events.

Dynorphin A-N/OFQ chimeric peptides have also been generated in an effort to examine the structure-activity relationships of these similar peptide agonists (Lapalu *et al.*, 1997). These studies suggested that the N-terminal portion of dynorphin A is essential for

high affinity binding and robust activity at KOR. In contrast, the C-terminal portion of N/OFQ appears to be fundamental for high affinity binding and activation of NOR (Lapalu *et al.*, 1997). These results imply that in spite of structural homology between dynorphin A and N/OFQ and the KOR and NOR receptors, the peptide ligand domains responsible for receptor interaction differ, which refutes the opioid message-address concept for the NOR-N/OFQ system (Lapalu *et al.*, 1997).

To further elucidate the differences in KOR and NOR functional architecture, KOR/NOR chimeras were generated (Mollereau *et al.*, 1999). A chimeric receptor was generated in which both N/OFQ and dynorphin A demonstrated binding affinity and functional potency equal to that observed at their respective wild-type receptors. This chimeric receptor contained the N-terminal portion of NOR to TMD 3 and the NOR second extracellular loop and all other KOR-derived domains (Mollereau *et al.*, 1999). These findings indicate that the mechanism of receptor binding and activation for N/OFQ and dynorphin A are distinct (Mollereau *et al.*, 1999), providing further evidence that N/OFQ-NOR interactions are not mediated via the "message-address" mechanism proposed for dynorphin A-KOR.

The exact mechanisms for N/OFQ binding and activation of NOR remain to be elucidated. However, a growing body of evidence suggests that these mechanisms differ from those utilized by dynorphin A and KOR and that the "message-address" hypothesis proposed for dynorphin A-KOR interactions does not apply to N/OFQ-NOR interactions. Although the binding and activation studies conducted on the NOR R299A mutant suggest that the mechanisms of binding and activation of NOR are separable, this data does not support the "message-address" hypothesis. The binding and activation mechanisms of N/OFQ at the NOR mutants appear to be at least partially dependent on charge interactions and not on specific N/OFQ domains. Moreover, most mutagenesis studies of N/OFQ and NOR refute the message-address hypothesis and strongly suggest that certain charged residues play a critical role in mediating N/OFQ-NOR interactions and activation. N/OFQ

truncation studies provide perhaps the most compelling argument against the "message-address" hypothesis. In the case of opioid peptides, the N-terminal amino acid sequence is sufficient to mediate opioid activity. Indeed, the enkephalins, the shortest opioid peptides composed of only five amino acids are capable of mediating opioid activity through high affinity binding to the δ-opioid receptor. In contrast with the opioid peptides, C-terminally truncated N/OFQ peptides shorter than $N/OFQ^{1\text{-}13}$ retain negligible binding affinity and functional potency for NOR. These data suggest that the N-terminal amino acids of N/OFQ are not sufficient to constitute the putative "message" portion of the peptide. Taken together, the data strongly refute the "message-address" hypothesis for NOR-N/OFQ interactions. Therefore, future studies should be designed to further examine the importance of receptor-ligand charge interactions rather than attempting to apply the "message-address" hypothesis to this system. These studies should include mutagenesis of charged residues of both NOR and N/OFQ and evaluation of the pharmacological and physiological interactions between mutant receptors and ligands.

Summary and Future Directions

The numerous functions attributed to N/OFQ suggest it is an important neuropeptide. Given the sequence homology of N/OFQ and NOR with opioid peptides and their receptors, respectively, it is tempting to speculate that the N/OFQ system is intimately involved in regulation of the opioid system and vice versa. Since N/OFQ appears to be involved in the modulation of many of the same behavioral effects as opioid peptides, including stress and pain perception, the anti-opioid theory for N/OFQ function is an intriguing idea. We developed a radioimmunassay (RIA) for N/OFQ in an effort to examine some of the behavioral functions of the peptide. Using the RIA we performed a survey of N/OFQ tissue distribution and investigated the possible role of N/OFQ in the activity of the hypothalamic-pituitary-adrenal (HPA) axis. The results of these studies

provide further evidence for the involvement of N/OFQ in the response to stress. However, the function of N/OFQ in the response to stress appears to be independent of interaction with the opioid system.

An interesting feature of the N/OFQ peptide is the putative function of its metabolic products. The data collected by others and by our group suggest that N/OFQ metabolism has behavioral consequences *in vivo* and affects *in vitro* experiments. N/OFQ peptide has been shown to be subject to metabolism when inject *in vivo* or in the presence of cultured cell lines (Monteil *et al.*, 1997; Sandin, *et al.*; Suder *et al.*; Vlaskovska *et al.*, 1999). Co-administration of N/OFQ and the protease inhibitors endopeptidase 24.15 and aminopeptidase N potentiates N/OFQ mediated behavioral effects, suggesting that N/OFQ metabolism appears to be attenuated by these protease inhibitors (Noble and Roques, 1997). Moreover, the putative N/OFQ metabolic product N/OFQ1-6 produces behavioral responses in the hot plate test when injected i.c.v. in rats (Suder *et al.*, 1999). We found that the tritiated N/OFQ peptide is intrinsically labile and may be subject to degradation even in storage conditions. Furthermore, in the presence of cellular material tritiated N/OFQ appears to be subject to enzymatic cleavage, which could not be completely blocked by various combinations of protease inhibitors. In spite of this enzymatic cleavage, we established a reproducible assay for binding of tritiated N/OFQ to NOR expressed in cellular membranes.

With the development of a reliable binding assay, we examined the molecular pharmacology of NOR-N/OFQ interactions. Mutant forms of NOR were created via site-directed mutagenesis. We hypothesized that specific NOR amino acids form a putative intrahelical salt-bridge linkage that is involved in receptor activation. Disruption of this salt-bridge linkage by site-directed mutagenesis was thought to result in a constitutively active conformation of NOR. This hypothesis does not appear to be correct for activation of NOR, however, the results of our site-directed mutagenesis studies suggest that NOR-N/OFQ charge interactions are important for receptor binding and activation. These

experiments have begun to elucidate the molecular pharmacology of N/OFQ binding and the functional activation of NOR.

Our studies of N/OFQ pharmacology and metabolism are important for the establishment of reliable pharmacological protocols. Our results should set the groundwork for future studies that might include determination of the mechanisms of N/OFQ-NOR interaction. Furthermore, the data presented in this dissertation will be useful background for the study of putative N/OFQ metabolic products and their possible interaction with NOR or other putative receptor sub-type(s). Identification and tissue distribution of NOR-related receptors may suggest behavioral roles of putative N/OFQ-derived peptides and may explain the myriad of behavioral functions attributed to N/OFQ.

A major limiting factor in the characterization of the N/OFQ-NOR system is the lack of a selective receptor antagonist. Such an antagonist will be imperative to validate experimental data and explore *in vivo* function of N/OFQ acting specifically at NOR. The experiments presented here represent an initial characterization of N/OFQ in terms of some of the physiological functions, peptide stability and interactions at NOR. These data will contribute to the groundwork to further characterize NOR and N/OFQ in the future.

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