Pharmacological and Behavioral Analysis of Nociceptive Modulation of Rat PrePro-Orphanin FQ/ Nociceptin Fragments in the Amygdala of Rats

by

Randi M. Shane

A dissertation submitted to the Graduate Faculty in Psychology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2002

This manuscript has been read and accepted for the Graduate Faculty in Psychology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

8/1/02
Date

Chair of Examining Committee

5/8/02
Date

Executive Officer

Dr. Richard J. Bodnar

Dr. Jeffrey M. Halperin

Dr. Grace C. Rossi

Supervisory Committee

The City University of New York

Abstract

PHARMACOLOGICAL AND BEHAVIORAL ANALYSIS OF NOCICEPTIVE MODULATION OF RAT PREPRE-ORPHANIN FQ/NOCICEPTIN FRAGMENTS IN THE AMYGDALA OF RATS

By

Randi M. Shane

Advisor: Professor Richard Bodnar

Orphanin FQ/nociceptin (OFQ/N) is a heptadecapeptide that binds with high affinity to the ORL-1/KOR-3 opioid receptor clone, yet binds poorly with traditional opioid receptors. OFQ/N previously exhibited a complex functional profile with relation to nociceptive processing, displaying pro-nociceptive properties in some studies, acting as an inhibitor of stress-induced analgesia in others, yet producing both spinal and supraspinal antinociceptive action in other studies. Prepro-Orphanin (ppORPH), the precursor gene for OFQ/N, contains several paired basic amino acids suggesting that it may be responsible for the production of additional biologically active peptide fragments. The first two studies of this dissertation examined the antinociceptive actions of rppOFQ/$N_{135-151}$ and two of its truncated fragments, rppOFQ/$N_{135-145}$ and rppOFQ/$N_{135-141}$, together with another biologically-active fragment of ppOrph, rppOFQ/$N_{154-181}$, following microinjection into the amygdala. Since the amygdala has been implicated in both antinociceptive and stress-related responses, and possesses a dense distribution of ORL-1 receptors, it was hypothesized that OFQ/N would exhibit its antinociceptive and potential hyperalgesic effects in the amygdala. rppOFQ/$N_{135-151}$, its shorter-chained active fragments, rppOFQ/$N_{135-145}$ and rppOFQ/$N_{135-141}$, as well as rppOFQ/$N_{154-181}$ each

produced antinociception as measured by reactivity to high-intensity radiant heat. In contrast to traditional μ and κ opioids and β-endorphin, none of the OFQ/N fragments in the amygdala exhibited antinociception on a test measuring shock reactivity. These ppOrph fragments failed to produce hyperalgesia as measured by reactivity to lower-intensity radiant heat. Therefore, OFQ/N fragments exert only antinociceptive responses in the amygdala with quantitative and qualitative differences relative to traditional opioid agonists. To establish whether OFQ/N-induced-antinociception is mediated by traditional opioids, the effects of systemic or intracerebral pretreatment with general or selective opioid receptor subtype antagonists was examined upon OFQ/N-induced antinociception elicited from the amygdala. This antinociceptive response was significantly reduced by general, μ, κ, and δ opioid antagonists, indicating an intrinsic circuitry within the amygdala involving these receptors and the ORL-1 receptor. The final study evaluated regional interactions between the amygdala and ventrolateral periaqueductal gray (vlPAG) in mediating rppOFQ/$N_{135-151}$-induced antinociception. OFQ/N-induced antinociception elicited from the amygdala was blocked by pretreatment with general and δ opioid antagonists in the vlPAG, whereas OFQ/N-induced antinociception elicited from the vlPAG was blocked primarily by pretreatment with general opioid antagonists in the amygdala. These studies indicate that OFQ/N appears to utilize a similar anatomical and functional pathway to that of the traditional opioid agonists in eliciting antinociceptive responses, and that its hyperalgesic properties are in all probability mediated by different supraspinal circuits.

Acknowledgements

I would like to thank all the members of my dissertation committee, Dr. Jeffrey Halperin, Dr. Robert Ranaldi and Dr. Alan Gintzler for their wisdom, guidance and expertise. I would especially like to acknowledge Dr. Grace Rossi who gave up so much of her time and energy to help me organize my thoughts and formulate my research question's, she is truly a role model. Dr. Richard Bodnar, to whom I owe more than just a thank you, it is to his credit and tenacity that this dissertation has come to be. He is a man of great intelligence and kindness and will continue to be an inspiration to me even beyond my academic life. And finally, my family, my sisters Elana and Sandra and brothers Avi and Alex, and especially my parents, Stewart Shane, Tamara and Nathan Braun, it is because of your love and support that I was able to achieve what I have and so it is to you that I dedicate this dissertation. Thank you!

Table of Contents

List of Tables

List of Figures

List of Abbreviations

βEND: β-endorphin, endogenous opioid peptide

βFNA: β-funaltrexamine, general μ receptor antagonist

BLA: basolateral nucleus of the amygdala

BNST: bed nucleus of the stria terminalis

Ce: central nucleus of the amygdala

CNS: central nervous system

CRF: corticotropin-releasing factor

DALCE: D-Ala^2, Leu^5, Cys^6-enkephalin, selective δ_1 receptor antagonist

DAMGO: D- Ala^2, met Phe^4, Gly(ol)^5-enkephalin, selective μ receptor agonist

DLF: dorsolateral funiculus

DOR-1: δ opioid receptor clone

DPDPE: D-Pen^2, D-Pen^5-enkephalin, selective δ_1 receptor agonist

i.c.v.: intracerebroventricular

i.t.: intrathecal

KOR-1: κ opioid receptor clone

KOR-3: κ_3 opioid receptor clone

LC: Locus Coeruleus

MOR-1: μ opioid receptor clone

Nacc: nucleus accumbens

NalBzOH: naloxone benzoylhydrazone, selective κ_3 receptor agonist

NTI: naltrindole, selective δ receptor antagonist

NTII: naltrindole-5'-isothiocyanate, selective δ_2 receptor antagonist

NTX: naltrexone, general opioid antagonist

NBNI: nor-binaltorphamine, selective κ receptor antagonist

NRM: nucleus raphe magnus

NRGC: nucleus reticularis gicantocellularis

NTS: nucleus tractus solitarius

ORL-1: orphanin opioid receptor clone

OFQ/N: orphanin FQ/nociceptin, endogenous opioid peptide

PVN: paraventricular nucleus of the hypothalamus

POMC: proopiomelanocortin, endogenous opioid peptide precursor

PpORPH: PrePro-orphanin, endogenous opioid peptide precursor

ProDYN: pro-dynorphin, endogenous opioid peptide precursor

ProENK: pro-dynorphin, endogenous opioid peptide precursor

RVM: rostroventral medulla

SIA: Stress-induced analgesia

vlPAG: ventrolateral periaqueductal gray

VTA: ventral tegmental area

2DG: 2-deoxy-D-glucose, antimetabolic agent

CHAPTER 1. INTRODUCTION

The family of opioid peptides, identified following the characterization of the opiate receptor, plays a major role in the control of nociception among a number of other behaviors. The existence of opioid receptors was first validated following demonstration of high-affinity, saturable, and stereospecific binding of opiate alkaloid compounds (Terenius, 1973; Simon et al., 1973; Pert and Snyder, 1973), such as morphine (Archer, 1998; Rees and Hunter, 1990), on brain membranes. Opiate drugs produce their strong antinociceptive and addictive actions by mimicking endogenous opioid peptides, and it is believed that over-activation of their receptors and interference with their signal transduction pathways are partly responsible for their adverse side effects.

The postulated existence of at least three opioid receptor classes was established using biochemical binding, pharmacological and bioassay techniques. These traditional μ, δ, and κ opioid receptors display different pharmacological profiles (Goldstein and Naidu, 1989) and anatomical distributions (Mansour et al., 1987). In vivo assays have demonstrated overlapping but distinct biological actions for μ, δ, and κ opioid agonists. Thus, in addition to their commonly-implicated roles in antinociceptive processes, the three traditional opioid receptors have been associated with distinguishable responses to painful stimuli, variable potential for dependence, as well as distinct and sometimes antagonistic mood-altering, autonomic, and neuroendocrine effects (Millan, 1990; Rapaka and Porreca, 1991).

Soon after the discovery of opiate receptors, putative endogenous ligands for these receptors were identified, and were surprisingly (at the time) found to be peptides rather than classic amino acid-based neurotransmitters. The first members of this family

to be characterized were Met- and Leu-enkephalin, isolated from the brain where they are most abundant (Hughes et al., 1975). These classic opioid peptides shared a common N-terminal sequence (NH^2-Tyr-Gly-Gly-Phe-Met/Leu), and were derived from larger precursor proteins that were translated from three different genes (Rossier, 1993; Young et al., 1993; Day et al., 1993). Subsequently, other traditional opioid peptides, such as β-endorphin (βEND) and Dynorphin were identified in the late 1970's and early 1980's (Goldstein et al., 1979; Guillamen et al., 1976). During the 1980's, a great deal of attention was paid to the development of selective opioid receptor subtype agonists and antagonists and the pharmacological and biochemical characterization of further subtypes of μ (μ_1, μ_2), δ (δ_1, δ_2) and κ (κ_1, κ_2, κ_3) opioid receptors (Pasternak & Wood, 1986). Opioid peptides were shown to play a major role in endogenous pain-controlling systems (Fields, 1993), were involved in the mediation of certain forms of stress-induced analgesia (Akil et al., 1984), modulated affective behavior including motivation and reward (DiChiara and North, 1992), affected locomotor activity (Cowan, 1993) and played a role in learning and memory (McGaugh et al., 1993). This group of opioid peptides was also found to modulate neuroendocrine physiology and controlled such autonomic functions as respiration (Shook et al., 1990), blood pressure thermoregulation (Adler and Geller, 1993), and gastrointestinal motility (Krommer, 1988).

Following the advent of molecular techniques used to identify the synthesis of receptors, the opioid receptors were finally cloned (Evans et al., 1992; Kieffer et al., 1992) and each of the major μ (MOR-1), δ (DOR-1), and κ (KOR-1) opioid receptor genes were identified (see reviews: Reisine and Bell, 1993; Uhl et al., 1994). In addition, a new "orphan" receptor (ORL-1) was characterized (Mollereau et al., 1994).

This led to the subsequent recent discoveries of a new generation of opioid peptides, including endomorphin (Zadina, 1997) and the subject of this dissertation, Orphanin FQ (OFQ) (Meunier et al., 1995) or Nociceptin, so named because in initial studies the peptide significantly lowered thermal latencies (Reinscheid et al., 1995). OFQ/N appears to be synthesized as part of a larger polyprotein precursor, Pre-Pro-Orphanin (ppOrph), which has multiple bioactive peptides, two of which are $rppOFQ_{135-151}$, and a second peptide downstream, $rppOFQ_{154-181}$. Thus, it is important to note that whereas the traditional opioid receptors were characterized initially by biochemical, pharmacological and behavioral evidence, and subsequently confirmed by molecular techniques, the ORL-1 receptor and its endogenous ligand, OFQ/N, were first identified by molecular techniques and the process by which they are being characterized biochemically, pharmacologically and behaviorally has only begun over the last seven years and continues. Therefore, the discovery of OFQ/N is an example of reverse pharmacology or functional genomics (see review: Meunier, 2000).

The descending pain-inhibitory system utilizes the endogenous opioid peptides to produce opioid inhibition of nociceptive input to the brain. The classic pathway subserving supraspinal opioid antinociception is mediated by neurons originating in the ventrolateral periaqueductal gray (vlPAG), which synapses in the rostral ventromedial medulla (RVM), including the nucleus raphe magnus (NRM), the nucleus reticularis giganotcellularis (NRGC) and the nucleus reticularis gigantocellularis pars α, and projects to the substantia gelatinosa of the spinal cord through the dorsolateral funiculus (see reviews: Fields and Basbaum, 1978; Basbaum and Fields, 1884; Bodnar, 2000). Forebrain structures have also been found to be involved in the antinociceptive process as

well, including the amygdala, which has a well-studied anatomical and functional relationship with the vlPAG (Tershner and Helmstetter, 1995).

The purpose of the present dissertation is to characterize the novel "orphan" ORL-1 receptor and its endogenous ligand, OFQ/N within the amygdala through analysis of pharmacological, anatomical, and mostly behavioral evidence. Four series of specific experiments are proposed.

The first study will evaluate the antinociceptive and hyperalgesic effects of rppOFQ/$N_{135-151}$ and two of its truncated fragments rppOFQ/$N_{135-145}$ and rppOFQ/$N_{135-141}$ in the amygdala in rats on both radiant heat (tail-flick) and shock (jump threshold) tests. These fragments were chosen because rppOFQ/$N_{135-151}$ and its two active truncated versions, rppOFQ/$N_{135-145}$ and rppOFQ/$N_{135-141}$ were found to produce antinociception in mice following ventricular administration, which was blocked by antisense probes targeted at exons 2 and 3, but not exon 1 of the ORL-1 clone (Rossi et al., 1997), and because their amino acid sequence suggests that they may be ideal splice sites to produce physiologically relevant peptides.

The second study will evaluate the antinociceptive and hyperalgesic effects of a related peptide, rppOFQ/$N_{154-181}$ in the amygdala of rats on both radiant heat and shock tests and to asses its interaction with classic opioid receptors using the antagonist naloxone. This peptide is at the end of the pre-pro Orphanin precursor gene. Initial studies with this and other related peptides found that it elicited similar antinociceptive effects to rppOFQ/$N_{135-151}$ when given i.c.v. in mice (Rossi et al., 1998). The present study using the rppOFQ/$N_{154-181}$ was part of a larger study examining at the effects of the peptide in other supraspinal sites (e.g., vlPAG, RVM and LC).

The third study will determine whether rppOFQ/$N_{135-151}$-induced antinociception in the amygdala can be blocked by pretreatment with equimolar doses of either general (naltrexone), μ (β-FNA), κ (NBNI) or δ (NTI) selective opioid receptor subtypes in rats. Autoradiographic and immunohistochemical techniques have shown that there is a dense distribution of ORL-1 as well as traditional opioid receptors in the amygdala (Letchworth et al., 2000; Mansour et al., 1987, 1994a, 1994b, 1994c, 1995a, 1995b, 1996). Previous studies (Pavlovic, et al., 1996; Pavlovic and Bodnar, 1998) found that antinociception elicited following amygdala microinjections of selective μ, κ_1 and β-endorphin is blocked by pretreatment of general and selective opioid antagonists within the same site. This present study would elucidate whether rppOFQ/$N_{135-151}$-induced antinociception acts through a similar mechanism to that of traditional opioid peptides, and whether this antinociception is mediated through a local amygdala circuit utilizing the traditional opiate receptors.

Antinociceptive responses following amygdala microinjections of traditional opioid agonists such as morphine, DAMGO, β-endorphin and U50 488H have been linked to an intrinsic pain-inhibitory pathway originating in the vlPAG in anatomical studies. (Krettak and Price, 1978; Beitz, 1982; Basbaum and Fields, 1984; Rizvy et al., 1991). Pretreatment within the vlPAG with lidocaine or microinjections of either general or selective μ or δ_2 opioid receptor antagonists significantly reduced the antinociception elicited by either morphine, β-endorphin, DAMGO or U50 488H in the amygdala (Pavlovic et al., 1996; Pavlovic et al., 1998; Helmstetter et al., 1998; Tershner and Helmstetter, 2000). The fourth study will evaluate whether rppOFQ/$N_{135-151}$-induced antinocicetpion elicited from the amygdala can be blocked by pretreatment with

equimolar doses of general (naltrexone), μ (β-FNA), κ (NBNI) and δ (NTI) opioid receptor subtypes in the vlPAG in rats, indicating the peptide utilizes a similar anatomical and functional pathway to that of the traditional opioid agonists. To examine the site-specificity of such antagonists effects, misplaced cannulae lateral and ventral to the vlPAG were also tested in antagonist studies. Finally, to examine reciprocal ascending influences, the ability of selective opioid antagonists administered into the amygdala were assessed for effects upon rppOFQ/$N_{135-151}$-induced antinociception elicited from the vlPAG.

To provide an underlying conceptual basis, background and rationale for these experiments, the following sections of the Introduction will examine: A) the traditional endogenous opioid peptide families, B) the traditional opioid receptor subtypes, C) opioid receptor genes, D) the ORL-1 receptor, E) the pre-pro-orphanin precursor, F) orphanin FQ/nociceptin (OFQ/N), G) behavioral effects of OFQ/N, H) the endogenous pain-inhibitory system, I) the role of the amygdala in opioid-induced antinociception and stress-induced analgesia (SIA) and J) a rationale for the present experiments.

A. Endogenous Opioid Peptide Families

By the early 1970's, it was suggested, but not confirmed, that opiates modulate pain perception by acting at putative receptors in the brain. In 1973, opioid receptors were discovered in the CNS (Terenius, 1973; Pert and Snyder, 1973; Simon et al., 1973), which immediately started the search for their endogenous ligands. Hughes et al., (1975) were the first to describe the structure of two opioid peptides, Leucine-enkephalin (Leu-Enk) and Methionine-enkephalin (Met-Enk), from pig and cow brains. Both peptides had potent agonist activity at opiate receptor sites producing a dose-related inhibition of

electrically-evoked contractions of the guinea-pig ileum and the mouse vas deferens (Hughes, Kosterlitz and Leslie, 1975). The characterization of β-endorphin and related peptides (Guillamin et al, 1976), and dynorphin and related peptides (Goldstein et al., 1979) soon followed which revealed a common amino acid Tyr-Gly-Gly-Phe core sequence present in each of these opioid peptides. It was later demonstrated that these peptides were derived from three distinct precursor molecules, or pro-hormones respectively, pro-enkephalin (Pro-Enk), pro-opiomelanocortin (POMC), and pro-dynorphin (Pro-Dyn), corresponding to the three distinct genes that were differentially distributed through the CNS (see review: Akil et al., 1984). It is now known that these three genes include approximately 20 peptides with opioid-like activity thereby acting as putative transmitters. Each of the opioid peptides is first synthesized as a part of one of the larger, inactive pro-hormone precursors. Cleavage at basic residues (e.g., arginine-arginine, lysyl-lysyl, or lysyl-arginine signal-specific cleavage) within these precursors results in the formation of biologically active neuropeptides (Young et al., 1993). A brief synopsis of each opioid peptide family now follows.

1.) POMC: POMC is the common precursor for beta-endorphin, its precursor beta-lipotropin (B-LPH), adrenocorticotropic hormone (ACTH) and related bioactive peptides (Mains, Eipper and Ling, 1977). $ACTH_{1-39}$ is further cleaved into α-melanocyte-stimulating hormone (α-MSH) and corticotropin-like intermediate lobe peptide (CLIP) (Brownstein, 1980; Mains, Eipper and Ling, 1977). β-endorphin, the 31 residue C-terminal component of β-LPH is generated from the post-translational process of β-LPH (Guillemin et al, 1976). $\beta\text{-endorphin}_{1-31}$ displays good affinity for μ and δ

opioid receptors and comparatively low affinity for κ opioid receptors (Mansour et al., 1995).

2.) Pro-Enk: Within the Pro-Enk precursor, a second major opioid gene product, are several active opioid peptides, including four copies of Met-Enk , and one of each copy of Leu-Enk, Met-Enk-Arg-Phe, and Met-Enk-Arg-Gly-Leu (Hughes et al., 1975; Kimura et al., 1980; Akil et al., 1984). Met- and Leu-Enk bind to the δ opioid receptor with high affinity, and to the μ receptor with good affinity (Lord et al., 1977; Chavkin et al., 1982). In contrast, they have markedly poor affinity for the κ opioid receptor, despite the presence of three copies of Leu-Enk in the Pro-Enk precursor (Mansour et al., 1995).

3.) Pro-Dyn: The Pro-Dyn precursor is cleaved to produce three Leu-Enk-containing peptides: α and β-neo-endorphin, dynorphin A (DynA), and dynorphin B (Goldstein et al., 1981). There are three peptide lengths of DynA that are biologically active, $DynA_{1-17}$, Dyn_{1-8} (DynB), and α- and β -neoendorphin (Hollt, 1986). $DynA_{1-8}$-induced antinociception can occur following co-administration of an endopeptidase 24.15 inhibitor (Chu and Orlowski, 1985) that was blocked by κ, but not μ, opioid antagonists (Kest et al., 1992), suggesting κ-receptor opioid activation with Dyn. A detailed discussion of Pre-Pro-Orphanin, the OFQ/N precursor gene, will follow in section E.

B. Opioid Receptor Subtypes

Since the discovery of opioid receptors almost 30 years ago (Pert & Snyder, 1973; Simon, et al., 1973; Terenius, 1973), bioassays, pharmacological, and biochemical approaches provided evidence for the existence of multiple receptor subtypes (Martin et al., 1976; Lord et al., 1977). Three distinct opioid receptors were proposed based on the lack of cross-tolerance among opioid receptor agonists: μ (morphine), κ

(ketocyclazocine), and σ (SKF 10,047) (Martin et al., 1976). The latter subtype was subsequently characterized as nonopioid because it was unaffected by the prototypical opioid antagonist, naloxone (Vaupel, 1983; Zukin, Brady, Slifer and Balster, 1984). Using bioassay and binding studies dissociations were found between morphine and endogenous enkephalin peptides since the enkephalins were more potent than morphine in the mouse vas deferens assay and less potent than morphine in the guinea pig ileum assay, leading to the suggestion of the existence of a δ opioid receptor (Lord et al., 1977). Based on the differential binding patterns and affinities between opioid receptors and opioid gene families, the μ opioid receptors were generally associated with POMC-derived opioid peptides, κ opioid receptors were generally associated with Pro-Dyn-derived opioid peptides, and δ opioid receptors were generally associated with Pro-Enk-derived opioid peptides (Bowen, et al., 1987; Cotton et al., 1984). However, as noted above, there is considerable cross-reactivity between different opioid peptides and receptors. Further, in contrast to the commonly-held assumptions of appositional synapses with transmitters and receptors in close proximity to each other, autoradiographic localization of opioid receptors and immunocytochemical localization of opioid peptides revealed receptor-peptide mismatches, providing evidence for modulatory actions by these peptides at distal sites (Herkenham et al., 1984). A brief discussion of each of the classic opioid receptors and their subtypes now follows.

1.) μ Opioid Receptors μ receptors are widely distributed throughout the brain, especially in areas related to pain control including the amygdala, PAG, dorsal raphe, locus coeruleus (LC), nucleus raphe magnus (NRM), nucleus reticularis gigantocellularis (NRGC) and the dorsal horn of the spinal cord (Mansour et al., 1988). Of the three major

types of opioid receptors, the μ receptor displays the highest affinity for morphine and other alkaloids with high abuse potential. The μ opioid receptor has been characterized pharmacologically using selective agonists, such as DAMGO, and the antagonists, β-FNA and CTOP. The μ receptor has been sub-characterized into μ_1 and μ_2 receptor subtypes based on pharmacological assays (see review: Pasternak & Wood, 1986). The μ_1 receptor binds morphine, ethylketocyclazocine, and enkephalin peptides with equally high affinity and is blocked by the antagonist, naloxonazine, while the μ_2 receptor binds morphine with high affinity and displays lower affinity for other opioid peptides and is naloxonazine-insensitive. Autoradiographic studies reveal similar, but not identical, distributions of μ_1 and μ_2 receptors (Goodman & Pasternak, 1985; Moskowitz & Goodman, 1985).

2.) δ Opioid Receptors Mansour et al., (1988) showed through autoradiographic studies that δ opioid receptor binding is densest in the olfactory tubercle, neocortex, neostriatum, nucleus accumbens (Nacc), and the amygdala. Pharmacological analysis of the δ receptor has utilized the general δ opioid agonists, DSLET and DADL (Lord et al., 1977; Mosberg et al., 1983), and the general δ opioid receptor antagonist, ICI 174864 and naltrindole (Cotton et al., 1984). The δ opioid receptor has been further classified into two subtypes, δ_1 and δ_2 (Negri et al., 1991). The δ_1 receptor subtype has been pharmacologically characterized by the selective agonist actions of DPDPE and the antagonist actions of DALCE, and a nonpeptide opioid antagonist 7-benzylidenenaltrexone (BNTX) (Vanderah et al., 1994; Portoghese et al., 1992). The δ_2 receptor subtype has been pharmacologically characterized by the selective agonist actions of D-Ala^2-deltorphin II and the antagonist actions of naltrindole 5'-isothiocyanate

(NTII) and the naltrindole analogue, Naltiben (NTB) (Vanderah et al., 1994). The effects of δ_1 and δ_2 agonists and antagonists have also been dissociated from each other in antinociceptive assays (Mattia, et al., 1991; Jiang et al., 1991).

3.) κ Opioid Receptor The distribution of κ opioid receptor indicate dense binding in the striatum, Nacc, amygdala, hypothalamus, neural lobe of the pituitary, median eminence and the nucleus tractus solitarius (NTS) and moderate binding in the PAG, NRM, other raphe nuclei, spinal trigeminal nucleus and the spinal cord (Mansour et al., 1988). Selective agonists and antagonists have been used to distinguish multiple κ receptor subtypes. The κ_1 receptor has been characterized using the selective agonist U50 488H (VanVoigtlander, Lahti and Ludens, 1983) and the antagonist norbinaltorphamine (NBNI: Portoghese, Lipkowski and Takemori, 1987). The existence of a U50 488H-insensitive κ_2 binding site was first demonstrated (Zukin et al., 1988) by examining the binding of [^{3}H]ethylketocyclazocine in the rat brain. Unfortunately, their pharmacological actions remain unknown. Another U50 488H-insensitive binding site (κ_3) was identified using naloxone benzoylhydrazone (NalBzoH), a novel opiate derivative (Clark, Liu, Price, Leison, Howard, Pack, Hahn and Pasternak, 1989, 1990; Gistrak, Paul, Hahn and Pasternak, 1990). Interestingly, opioid peptides, like opiate drugs, can have functionally antagonistic effects upon each other. For example, low doses of nalorphine, a drug which elicits its antinociceptive effects through a κ receptor subtype (Paul et al., 1991), antagonizes the antinociception produced by fixed doses of morphine (Lasagna et al., 1954; Houde et al., 1956). This is of importance because OFQ/N was also initially proposed as an anti-opiate. A detailed discussion of this issue will follow in section F.

C. Opioid Receptor Genes

In the 1980's, there was a merging of neuroscientific and molecular biological techniques allowing neurotransmitter receptors to be cloned and their genes identified. The first cDNA encoding of an opioid receptor was isolated simultaneously by two laboratories in 1992. This cDNA was identified by expression cloning in mammalian cells (Evans et al., 1992; Kieffer et al., 1992), and encoded a mouse δ opioid receptor. The cloned receptor belonged to the family of seven trans-membrane G-protein-coupled receptors (Kenakin, 1997). Homology cloning strategies led to subsequent cDNA encoding of μ and κ opioid receptors. The first identification of an opioid receptor cDNA has opened the way to the identification of the opioid receptor gene family and provided molecular tools to study opioid receptor diversity and function both in vitro and in vivo (Uhl et al., 1994; Pasternak and Standifer, 1995).

Thus the opioid receptor family was characterized at the molecular level. At present, this family consists of three homologous genes, MOR-1, DOR-1, and KOR-1, encoding μ, δ, and κ opioid receptors, respectively. The concept of multiple receptor subtypes within each μ, δ, and κ receptor class has emerged from accumulating pharmacological data. The availability of numerous synthetic opiates and their use in biological assays has indicated a possible heterogeneity within each receptor class. Thus, the existence of a δ_1- and δ_2, μ_1- and μ_2-, as well as κ_{1a}-, κ_{1b}, κ_2-, and κ_3-opioid receptors, distinguishable by highly specific ligands, has been proposed (Traynor, 1989; Traynor and Elliot, 1993; Pasternak, 1993). Although only one receptor has been cloned for each class, its relationship to the pharmacologically defined opioid receptor subtypes is unclear. Studies using antisense mapping suggest that these opioid receptor subtypes are

a result of alternative splicing of the opioid receptor clones (Rossi et al., 1995). Over ten alternative splice variants for the MOR-1 clone have been identified (Pan et al., 2001); however, how these multiple isoforms correlate with the pharmacologically distinct μ-receptor subtype is not known. It is of note that low stringency hybridization procedures have also led to the isolation of an opioid receptor-like cDNA with strikingly high homology to the three opioid receptor clones (Mollereau et al., 1994). This putative receptor clone, expressed in mammalian cells, does not encode an opioid binding site and therefore remains as a brain orphan receptor, and will be discussed in the next section.

Protein sequences of the cloned human μ, δ, and κ opioid receptors indicates the presence of seven putative trans-membrane domains, characteristic of G-protein-coupled receptors (Probst et al., 1992). The three intracellular loops; and a short region of the C-terminal tail are almost identical across subtypes. Little or no homology is found in the extracellular loops or in the N- and C-terminal tails. Intracellular domains of the receptors are implicated in receptor signaling and regulation. Extracellular loops, which differ in sequence between opioid receptor types, play a role in discriminating μ, δ, and κ opioid ligands. This suggests a possible functional organization in which a similar opioid-binding pocket, formed by the seven putative alpha-helixes, might exist within all three opioid receptors, while their extracellular loops would discriminate various classes of opioid ligands and define μ-, δ-, and κ-selectivity (Kieffer, 1995).

The strong sequence homology in the third intracellular loop as well as in the short amino acid stretch of the C-terminal tail suggests that the three opioid receptor types interact with similar G-proteins (Strader, 1994). This observation is consistent with the ability of all three receptors to activate almost identical intracellular effectors and

transduction pathways. The lack of sequence identity in the C-terminal tail, a region which has been shown critical for desensitization of G-protein coupled receptors, raises the possibility of distinct regulation patterns for μ, δ, and κ receptors by intracellular components. Coupling properties are similar for all three receptor types whereas the regulation of their activity may differ widely (Reisine, 1995).

Opioid receptors are coupled to G proteins which in turn modulate intracellular effectors. All three receptor classes have been shown to inhibit adenylate cyclase, decrease the conductance of voltage-gated Ca++ channels or activate inwardly rectifying K+ channels. Any of these effects ultimately leads to inhibition of neuronal activity. Inhibition of the cAMP pathway has been demonstrated in various cell lines and for all three cloned opioid receptors (North, 1986).

D. The ORL-1 Receptor

Another opioid receptor-related cDNA, with high-sequence homology to the three cloned opioid receptors (MOR-1, DOR-1, and KOR-1) was isolated from rat, mouse, and human brain cDNA libraries (reviewed in Kieffer, 1997). Attempts to measure specific opioid binding to the recombinant receptor were unsuccessful. It did not bind any of the known opiate ligands with high affinity. This cDNA was named the opioid-like orphan receptor (ORL-1: Mollereau et al., 1994). Then, two groups subsequently reported the isolation of an endogenous ligand for this receptor (Meunier et al., 1995; Reinscheid et al., 1995). This heptadecapeptide, called orphaninFQ or nociceptin, displays structural similarities to opioid peptides but does not act at opioid receptors as detailed by its lack of pharmacological effects using opioid antagonists. With the discoveries of MOR-1, DOR-1, and KOR-1 genes, this confirmed and validated all of the pre-existing ideas

about opiate receptors and their heterogeneity. However, the discovery of the ORL-1 gene by Mollereu, et al., (1994) created a paradigm shift in the understanding of opiate pharmacology since it led not only to a new receptor (ORL-1) but to a new opioid peptide family (Pro-Nociceptin). Early studies that will be detailed below suggested anti-opioid and anti-stress actions of the novel receptor and its peptides, further distinguishing them from traditional opioids. Therefore, this and the next sections will discuss the "orphan" receptor, ORL-1, the novel gene family, Pre-Pro-Orphanin (ppOrph), and ORL-1's endogenous ligand, OFQ/N.

The discovery of the ORL-1 receptor was driven by advances in molecular biology. After cloning the δ opioid receptor (DOR-1) (Evans et al., 1992; Kieffer et al., 1992), probes based on its structure were used to screen cDNA libraries for homologous receptors. In addition to the cloning of the μ and κ opioid receptors, this procedure resulted in the cloning of an "orphan" opioid receptor (Bunzow et al., 1994;Mollereau et al., 1994). The ORL-1 receptor shares many characteristics with the traditional opioid receptors. It is similar in length to opioid receptors: ORL-1 (367 amino acids), MOR-1 (398 a.a.), DOR-1 (380 a.a.), and KOR-1 (372 a.a.). The overall amino acid similarity between ORL-1 and the traditional opioid receptor from various species is about 65% (Chen, et al., 1994), whereas amino acid identity is about 50% (Bunzow, et al., 1994). The ORL-1 receptor also belongs to the super-family of seven trans-membrane G-protein-coupled receptors. Alignment of the amino acid sequences of the MOR-1-, DOR-1-, KOR-1-, and ORL-1 receptors reveal that the most highly conserved regions are the trans-membrane helices and cytoplasmic loops displaying about 80% similarity (Chen, et al., 1994) and 65% identity (Bunzow et al., 1994). Thus, the major differences among

opioid receptors are in their extracellular and intracellular domains reflecting differential binding and signal transduction mechanisms.

The distribution of the ORL-1 receptor was examined at the mRNA levels using *in situ* hybridization, and was found to be similar to that of traditional opioid receptors, especially in neuroanatomical areas involving pain processing. In rodents, moderate to high levels of mRNA were found in the cerebral cortex, septum, diagonal band of Broca, subfornical organ, nucleus accumbens, amygdala, thalamus, hypothalamus, suprachiasmatic nucleus, hippocampus, periaqueductal gray, dorsal raphe, locus coeruleus, dorsal horn of the spinal cord, and dorsal root ganglia (Bunzow,et al., 1994; Mollereau et al., 1994). Extensive immunohistochemical mapping of the ORL-1 receptor shows a similar distribution pattern to that of the mRNA, indicating that the ORL-1 receptor is expressed predominantly in local-circuit neurons (Anton, et al., 1996), suggesting local modulatory actions. Despite the structural and anatomical similarities between the ORL-1 receptor and the traditional opioid receptors, opioid ligands do not bind specifically or with high affinity to ORL-1 (Bunzow, et al., 1994).

E. Pre-Pro-Orphanin (ppOrph) Precursor

The novel peptide, OFQ/N, appears to be synthesized as part of a larger polyprotein precursor (187 amino acids), Pre-Pro-Orphanin (ppOrph) (see reviews: Meunier, 1997; 2000). It shares several properties with POMC, Pro-Dyn, and Pro-Enk, and it has been suggested that all four share a common evolutionary origin (Mollereau, et al., 1996). For example, ppOFQ shares 27% similarity with Pro-Dyn, 25% with Pro-Enk, and 13% with POMC (Nothacker, et al., 1996).

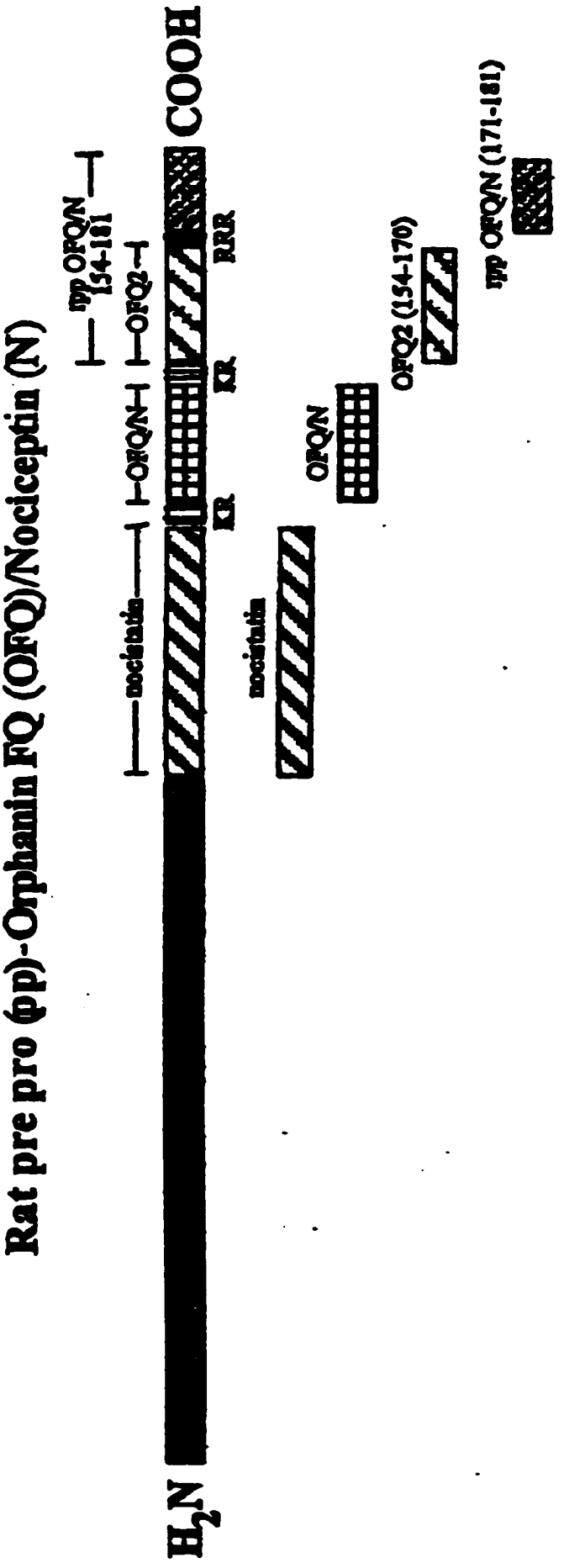
The ppOFQ gene contains only one copy of the OFQ/N sequence (residues 135-151) flanked by pairs of basic amino acid residues. Whenever there is a double amino acid (i.e., Lys-Lys or Arg-Arg) this is a potential cleavage site for a functional peptide (see review: Pasternak, 1993). In addition to the cleavage sites flanking rppOFQ/$N_{135-151}$, ppOFQ contains further basic amino acid proteolytic cleavage sites, suggesting that it may also be a precursor of other bioactive peptides such as rppOFQ-$2_{141-157}$ and rppOFQ/$N_{154-181}$, as well as two truncated fragments of rppOFQ/$N_{135-151}$, including rppOFQ/$N_{135-145}$ and OFQ/$N_{135-141}$; these points will be discussed in a later section. Because ppOFQ contains only one copy of OFQ/N, and potentially contains other biologically active peptides with other functions, it may act more like POMC than the other opioid precursors (Mollereau, et al., 1996) (Figure 1).

Extensive precursor mapping studies have been done using *in situ* hybridization showing that the location of ppOFQ is in general agreement with the ORL-1 receptor (Neal, et al., 1999; Boom, et al., 1999). Immunohistochemical antisera to ppOFQ label different fiber systems in several pain-modulatory areas , including the superficial dorsal horn, sensory trigeminal complex, the PAG and the amygdala (Riedl, et al., 1996).

F. Orphanin FQ/Nociceptin (OFQ/N)

Two groups simultaneously reported the isolation and identification of a novel peptide from the brain with high affinity for the ORL-1 receptor. Orphanin FQ or nociceptin (OFQ/N) (Reinscheid et al., 1995; Meunier et al., 1995) is a heptadecapeptide with the sequence Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asp-Glu which is structurally similar to Dynorphin A. However, unlike the opioid peptides with their N-terminal Tyr-Gly-Gly motif, OFQ/N has a Phe-

Figure 1. A schematic drawing of the Pre-Pro-Orphanin FQ/Nociceptin gene containing peptide location of rppOFQ/$N_{135-151}$, rppOFQ/$N_{154-181}$, OFQ/N2 and nocistatin.



Gly-Gly sequence. OFQ/N also contains two pairs of basic amino acid residues, raising the possibility that the peptide may be further processed to either rppOFQ/N $_{135-145}$ or OFQ/N$_{135-141}$ (Rossi et al., 1997).

The wide distribution of OFQ/N in the CNS has been confirmed by several groups. The results of alternative approaches (e.g., autoradiography and binding studies) are all in agreement as to the localization of this endogenous peptide. OFQ/N has been shown to influence nociception and locomotion (Meunier et al., 1995; Reinsheid et al., 1995; Florin et al., 1996), which is consistent with the anatomical localization of OFQ/N and its receptor, ORL-1. With regard to the processing of nociceptive information, dense levels of OFQ/N have been reported in the superficial layers of the dorsal horn, the sensory trigeminal complex, the PAG, amygdala and the raphe nuclei (Schulz, et al., 1996; Riedl, et al., 1996, Letchworth et al., 2000). Several areas of the CNS involved in motor control showed the presence of OFQ/N. It is densely distributed in the motor cortex and subcortical motor nuclei in the mesencephalon including the subthalamic nucleus, the substantia nigra and the red nucleus (Sim and Childers, 1997). High levels of OFQ/N in the cortex, hippocampus, and hypothalamus suggest that this system may be involved in memory and cognition (Sim and Childers, 1997). The traditional opioid peptides show high levels of binding in the amygdala and ventral tegmental area, which is consistent with the reinforcing effects of opioids (Ciccocioppo, et al., 2000). OFQ/N is apparently not reinforcing (Devine et al., 1996), which may be reflected by the low levels of OFQ/N binding in the nucleus accumbens (Sim and Childers, 1997). Although OFQ/N is related structurally to the classic opioid peptides, its anatomical distribution suggests that there may be some important functional differences.

G. Behavioral Effects of OFQ/N_{1-17}

1. Nociceptive Responses: Pharmacologically, OFQ/N has a complex series of actions. In the original papers on the isolation and characterization of OFQ/N, the authors showed that when OFQ/N was administered to the mouse by i.c.v. injection, the peptide produced a hyperalgesic response, i.e., a hypersensitivity to pain, measured by either the hot-plate (Meunier et al., 1995) or the tail-flick (Reinscheid et al., 1995) test. Hence, Meunier and co-workers gave this peptide the name "nociceptin," to highlight the seemingly paradoxical 'pro-nociceptive' action. However, further investigations have indicated that the effect of OFQ/N on nociceptive responses is far more complicated than simply lowering nociceptive thresholds. Further studies have shown either hyperalgesia, blockade of stress-induced analgesia, inhibition of opioid induced antinociception, allodynia, and even antinociception. Many factors have differed among these studies, such as species, route and sites of administration, the nociceptive test used, the methodological procedures, dose of OFQ/N administered, the time course of action, and the physiological state of the subjects. These differences clearly contribute to the various outcomes of OFQ/N. It should be noted here that it is not unusual for an opioid peptide to have such a wide spectrum of antinociceptive responses. For example, κ opioid agonists, such as Dynorphin, have been known to inhibit μ opioid-induced analgesia (Lasagna et al., 1954; Houde et al., 1956), as mentioned earlier in the paper. A brief discussion of the various nociceptive responses now follows.

<u>OFQ/N as a Hyperalgesic or Anti-Opioid agent:</u>

The first behavioral effect of OFQ/N to be described was a hyperalgesic response following i.c.v. injections in mice (Meunier et al., 1995; Reinscheid et al., 1995).

However, subsequent studies with additional controls showed that the i.c.v. injection itself caused an opioid-mediated stress-induced analgesia (SIA) that can be reversed by either naloxone or OFQ/N (Mogil et al., 1996a). Analgesia is typically assessed in the tailflick assay, one of the more common nociceptive tests used, and when used in naïve animals, it produces a prolonged tailflick latency. This type of nociceptive response is known as novelty-induced analgesia (Rochford and Dawes, 1993; Spreekmeister and Rochford, 2000) and auto-analgesia (Chance, 1980). Conversely, an increased sensitivity toward a nociceptive stimulus results in shorter latencies, or hyperalgesia. When mice were given i.c.v. saline injections, they displayed longer latencies to a lower-intensity nociceptive stimulus. OFQ/N reversed this form of SIA, and the resultant shorter latencies were interpreted as hyperalgesia. In addition, OFQ/N was shown to reduce antinociception induced by selective μ-, δ-, and κ-opioid receptor agonists (Mogil et al., 1996b). Analgesia induced by the μ agonist DAMGO, the δ agonist DPDPE, or the κ agonist U50 488H was blocked by i.c.v. OFQ/N-pretreatment as was morphine-induced analgesia in mice (Mogil et al., 1996a) and rats (Tian et al., 1997). However, there is considerable literature demonstrating that simultaneous administration of κ and μ agonists produce greater antinociception than μ agonists alone (Bodnar et al., 1991) and δ-μ combinations produce less antinociception than μ agonists alone (Porreca et al., 1984; 1987). Since κ and δ agonists produce antinociception alone, they were not proposed as "pro-nociceptive" or "anti-opioid."

One difference that may help explain why anticnociception was not observed in initial reports may be due to species and strain differences. It has been demonstrated that OFQ/N antinociception is dependent on the strain of mouse examined. CD-1 mice have

proven to be sensitive to a wide variety of antinociceptive, including opioids inactive in other strains. Thus, the sensitivity of these mice to OFQ/N is consistent with previous observations with other opioids (Mogil et al., 1996; Rossi et al., 1997).

OFQ/N as an Antinociceptive Agent:

Rossi et al., (1996) performed the initial study reporting a biphasic response following i.c.v. injections of OFQ/N comprising a brief period of hyperalgesia followed by a prolonged period of naloxone-sensitive antinociception in mice. The OFQ/N hyperalgesia gradually resolved over time, with tail-flick latencies continuing to increase significantly above their initial baseline levels, implying antinociception. In control studies, saline-treated mice did not demonstrate any significant changes in tail-flick latencies over time. When the mice were pretreated with diprenorphine, an opioid antagonist, no effect on the OFQ/N hyperalgesia was found; however, it completely reversed the antinociceptive effects of OFQ/N. The loss of antinociceptive activity and the persistent hyperalgesia implies that the tail-flick responses results from different hyperalgesic and antinociceptive systems in the CNS. Based on the diprenorphine sensitivity, the OFQ/N- induced hyperalgesia can be classified as nonopioid-mediated, while the antinociception is opioid-mediated. Important methodological differences could also account for these results. Instead of using naïve animals acutely exposed to the nociceptive stimulus and subject to antinociceptive actions of novelty or stress, these animals were habituated to the test procedures producing more normal nociceptive responses.

After the initial reports of hyperalgesia and reversal of opioid antinociception, reports of OFQ/N-induced antinociception began to emerge. Initial studies that detected

antinociception utilized the intrathecal (i.t.) route of administration with OFQ/N doses in the microgram (μg) range. King et al., (1997) detected a rapidly-appearing spinal antinociception in mice which was induced by OFQ/N and reversed by naloxone and the μ antagonist β-FNA (Jhamandas et al., 1998). Spinally-delivered OFQ/N was found to reduce flinching behavior in the formalin test, a model of inflammatory pain, in rats (Yamamoto et al., 1997), as well as reduce thermal hyperalgesia caused by paw injection of carrageenan (Yamamoto et al., 1997). Intrathecal administration of OFQ/N does not appear to antagonize morphine antinociception; rather it actually potentiates it through additive or multiplicative interactions (Tian et al., 1997; Jhamandas et al., 1998). Also, i.t. administration of OFQ/N potentiates electroacupuncture antinociception, which is presumably produced by the release of endogenous opioids (Tian et al., 1997). Further, OFQ/N was examined for its role in pregnancy-induced antinociceptive processes (Dawson-Basoa and Gintzler, 1997; Gupta et al., 2001) with spinal pretreatment with OFQ/N reducing the magnitude of pregnancy-induced antinociception. Pregnancy induces spinal dynorphin and kappa opioid receptor activity that can be reduced by pretreatment with OFQ/N (Gupta et al., 2001), suggesting that it is a negative modulator of spinal dynorphin release. However, the same type of reduction is induced by pretreatment with exogenous κ and δ agonists. Therefore, this suggests that these receptor subtypes sometimes act in an inhibitory manner relative to each other, and not in an "anti-opioid" mode.

A dichotomy was emerging in which supraspinal (i.c.v.) administration of OFQ/N produced hyperalgesia and antagonism of opioid antinociception, whereas spinal (i.t.) administration produces antinociception, and opiate-like action. It should be noted that

not all investigators found that spinal administration of OFQ/N induces antinociception Intrathecal injections of very low nanogram (ng) range doses of OFQ/N in mice causes allodynia, a pain-like response to innocuous stimulation, while even lower picogram doses elicits hyperalgesia and a decrease of nociceptive thresholds on the hot plate test (Okuda-Ashitaka et al., 1996; Hara et al., 1997). However, similar effects are also seen following spinal morphine administration. Neither the effects of morphine or OFQ/N at these low doses are naloxone-reversible (Hara et al., 1997). OFQ/N spinal-induced allodynia is blocked by prostaglandin, δ_2 and glycine antagonists (Minami et al., 1997), and the hyperalgesia is blocked by glycine and tachykinin NK1 receptor antagonists (Hara et al., 1997). One explanation for the opposing effects of spinally-administered OFQ/N at different doses may in fact reflect recruitment of distinct populations of neurons. Low doses (nanogram and picogram range) of i.t. OFQ/N may have diffused only superficially to cause allodynia and hyperalgesia, whereas higher doses (microgram range) may have reached deeper into the spinal cord to produce antinociception (see review: Meunier, 2000).

More recent studies have shown OFQ/N-induced antinociception following i.c.v. and i.c. injections. Rossi et al., (1997) examined OFQ/N antinociception in the tailflick assay following i.c.v. injections of the peptide. In this assay, baseline latencies typically range between 2 to 3 seconds and antinociception is defined as a doubling or greater of the baseline latency (Rossi et al., 1997). This assay is relatively insensitive to hyperalgesia. It was found that OFQ/N antinociception was dose-dependent, and remained sensitive to diprenorphine, naloxone, and naltrexone, confirming the general opioid nature of its actions. It is possible that the opioid sensitivity seen with OFQ/N

may reflect the activation of traditional opioids downstream of the ORL-1 receptor. However, this seems unlikely since antisense probes directed against the MOR-1, DOR-1, or KOR-1 opioid genes, a receptor knockdown procedure, respectively effectively blocked μ, δ, and κ_1-mediated antinociception, yet each of these probes had no effect on OFQ/N antinociception (Pasternak & Standifer, 1995; Pan et al., 1995; Rossi et al., 1995).

A second difference that may account for the contrasting effects of OFQ/N, i.e., hyperalgesia that is insensitive to opioid antagonism versus antinociception that is readily reversed by naloxone, naltrexone, and diprenorphine, (Rossi et al., 1997; Gintzler et al., 1997), may be a result of two distinct receptor mechanisms for the peptide. Such a model might argue that different receptor subtypes or splice variants of OFQ/N differentially mediate these effects. The best way to study this hypothesis would be to develop an antagonist for each ORL-receptor subtype. Antagonists have recently been discovered for OFQ/N; however, none of them seem to specify receptor subtypes like the selective traditional opioid antagonist naloxonazine (μ_1), DALCE (δ_1), or NTII (δ_2). However, there is another way to study the possible existence of multiple receptor subtypes, and that is by utilizing antisense oligodeoxynucleotides, a short-term knockdown of a receptor due to injection of nucleotides which can bind to a particular receptor's mRNA to delay their translation and enhance their degradation (Pasternak and Standifer, 1995). The use of antisense deoxynucleotides as a tool to study the possibility of multiple receptor subtypes for the ORL-1 clone was very elegantly demonstrated by Rossi et al., (1997). In this study, antisense probes targeting the first coding exon of the ORL-1 receptor blocks OFQ/N hyperalgesia without altering OFQ/N antinociception (Pasternak

& Standifer, 1995, Pan et al., 1995). However, hyperalgesia remains untouched by two antisense probes aimed against the second and third coding exons (Pasternak & Standifer, 1995; Pan et al., 1995), while OFQ/N-induced antinociception was completely blocked by the antisense probes. These results raise the possibility that the various effects of OFQ/N may be due to the peptide acting through multiple OFQ/N receptors created by splice variants.

A third possibility for the differential pharmacological actions of OFQ/N may be due to separate and distinct systems for pain perception. In a study by Rossi et al., (2002), rppOFQ/$N_{154-181}$, a second active peptide of ppOFQ and related to OFQ/N, was administered into four brain sites involved in pain-inhibitory processing: the amygdala, the vlPAG, the LC, and the RVM, to explore its antinociceptive and pronociceptive effects. Interestingly, in the four rat brain sites examined, rppOFQ$N_{154-181}$ produced potent antinociception. However, the antinociceptive effects in only two of the sites, the amygdala and the vlPAG, were naloxone reversible, while the other two sites, the LC and RVM displayed naloxone-insensitivity. In contrast, those sites that did not produce an opiate reversible antinociception, the LC and the RVM, showed significant pronociceptive effects, or hyperalgesia, whereas the amygdala and vlPAG did not. This double dissociation provides evidence for the evolution of at least two distinct systems for OFQ/N pain perception (opiate and non-opiate), which may be dependent on the supraspinal site of action at which OFQ/N may be released; this study is discussed in detail in the second specific aim.

OFQ/N has been well-established as a pain modulatory peptide; however, like other opioid peptides, it also has effects on other behaviors reviewed below, such as reward and addiction, stress and anxiety, locomotion, feeding, and learning and memory.

2. rppOFQ/$N_{135-151}$ and Reward and Addiction: In a test of conditioned place preference, in which chronic i.c.v. administration of OFQ/N was paired with a particular compartment of a shuttle box, OFQ/N failed to induce either place preference nor aversion, implying that the peptide is devoid of abuse potential (Devine et al., 1996). It did, however, block morphine-induced conditioned place preference following i.c.v. administration (Murphy et al., 1999). OFQ/N does no affect heroin self-administration in rats (Walker et al., 1998), but it does decrease alcohol consumption in genetically selected alcohol-preferring rats (Ciccocioppo et al., 1999).

There is some evidence for adaptation (upregulation) of the ORL-1 receptor system in the morphine-tolerant/dependent rodent. Mice that lack the prepro-OFQ gene through gene knockout have been tested for their ability to become tolerant to chronic morphine. These mice show a 50% reduction in the development of tolerance to chronic morphine, as compared with wild-type controls (Ueda et al., 1997). The fact that tolerance was only reduced by 50% in OFQ/N knockout animals suggests that although OFQ/N may play a role, it is not the only factor participating in tolerance and may not be the only opiate-modulating peptide involved (Harrison and Grandy, 2000).

3. rppOFQ/$N_{135-151}$ and Stress and Anxiety: OFQ/N has been proposed to have anxiolytic properties based on its effects in several behavioral tests of anxiety. In mice, i.c.v. injection of OFQ/N increases exploratory behaviors (Florin et al., 1996). The

actions of OFQ/N resemble that of diazepam and suggest that the peptide can decrease anxiety (Jenck et al., 1997).

4. rppOFQ/N$_{135-151}$ and Locomotion: OFQ/N has been shown to have an effect on locomotion; however, the exact nature of this effect remains controversial. Like other opioids (cf, in: Leventhal et al., 1996), studies have demonstrated both increases and decreases in locomotion. I.c.v. injections of low doses (10-100 ng) of OFQ/N in mice stimulate locomotor activity (Florin et al., 1996), whereas higher doses (>1 μg), decreases such activity, causing ataxia (Reinscheid et al., 1995). I.c.v. injections of the peptide into rats (20-200 μg) induces pronounced long-lasting motor impairments including loss of balance and muscle tone (Devine et al., 1996). The ability of OFQ/N to impair locomotion may be supraspinal since rats that have received i.t. injections of the peptide (1-20 μg) do not show signs of motor deficiency (Xu Xj et al., 1996).

5. rppOFQ/N$_{135-151}$ and Feeding: OFQ/N has stimulatory effects on feeding behavior such that i.c.v. injections of 1-10 nM of the peptide increases feeding in satiated rats. This effect resembles that of other opiates and is naloxone reversible (Pomonis et al., 1996). An involvement of both ORL-1 and other opioid receptors is also implied by studies in which hyperphagia following i.c.v. injection of OFQ/N is antagonized by either antisense probes directed against ORL-1 receptor mRNA, and is blocked by naloxone (Leventhal et al., 1998). Injection of OFQ/N into the shell of the nucleus accumbens or the ventromedial hypothalamus produce significant increases in food intake in satiated and unsatiated rats (Stratford et al., 1997).

6. rppOFQ/N$_{135-151}$ and Learning and Memory: A role for OFQ/N in spatial learning has been suggested by studies in which the peptide was injected into the

hippocampus and by studies using the ORL-1 gene knockout mice. Microinjection of OFQ/N into the CA3 region of the hippocampus in rats markedly impaired spatial learning without affecting swimming ability on the Morris water task, in which subjects must learn the location of submerged platforms (Sandin et al., 1997). Greater learning ability and memory capacity is observed in mutant mice lacking the ORL-1 receptor gene, compared with wild type control animals (Mamiya et al., 1998). The deleterious effects of OFQ/N on learning may be correlated with the ability of the peptide to inhibit long-term potentiation (LTP) in hippocampal slices (Yu et al., 1997). Mice lacking the ORL-1 receptor gene exhibit substantially larger LTP that do wild type control animals (Manabe et al., 1998).

Since its discovery in 1995 (Meunier et al., 1995; Reinscheid et al., 1995), OFQ/N has been established as a modulatory peptide with multiple actions in the nervous system. The widespread localization of OFQ/N and the ORL-1 receptor throughout the CNS correlates with the peptide's diverse actions. Behaviorally, OFQ/N has been reported to affect nociception, reward and addiction, stress and anxiety, locomotion, feeding, and learning and memory. However, it is not unusual for a peptide to exhibit such diverse effects (Kastin et al., 1987).

A striking feature of OFQ/N and its ORL-1 receptor is their similarity, based on sequence homology, to opioid peptides and their receptors, respectively. Many of the diverse actions of OFQ/N such as antinociception, stress responses, and feeding, are known to involve endogenous opioids (Akil, 1984; Bodnar, 1996). There are several relationships between OFQ/N and opioid peptides. First, OFQ/N could act in a similar manner to the other opioids, possibly as a fourth class of the opioid receptor family. This

idea stems from their sequence homology and presumed evolutionary relationship, and similarities in their anatomical localization and signal transduction mechanisms (Harrison and Grandy, 2000). Alternatively, OFQ/N could function in an opposite manner then that of the traditional opioids, so that their actions oppose one another. Finally, the actions of OFQ/N and the traditional opioids could be connected to one another such that the action of one induces the production and/or action of the other (Harrison and Grandy, 2000). This latter explanation holds the most resonance based on the most recent literature reviewed, and is the focus of the present studies examining antinociceptive interactions. The next section will briefly review endogenous pain-inhibitory pathways.

H. Endogenous Pain Inhibitory Pathway

Neural circuits in the mammalian nervous system modulate ascending transmission of noxious information when activated by environmental stressors, or by direct stimulation (Fields & Basbaum, 1978; Basbaum & Fields, 1984). Several discrete neuronal sites have been implicated as being a part of this descending pain-inhibitory pathway, such as the amygdala, vlPAG, LC, RVM, and a number of spinal sites (Fields and Basbaum, 1978; Basbaum and Fields, 1984; Helmstetter, 1992; Watkins et al., 1993). Opioids can modulate pain perception at both the spinal and supraspinal levels of the neuroaxis. The following sections briefly highlight a large literature on these subjects.

Spinal Systems: The ultimate effect of stimulation of supraspinal sites involved in pain control is inhibition of spinal cord neurons. The descending inhibitory effects are exerted on neurons in the substantia gelatinosa (SG), lamina II of the dorsal horn of the spinal cord (see review: Basbaum & Fields, 1984). The SG contains neurons that are responsive to noxious and non-noxious stimuli (Light et al., 1979; Bennett et al., 1980).

Enkephalin and dynorphin are contained within SG neurons, indicating an opioid-mediated effect of nociceptive inhibition of spinal cord neurons (Hunt et al., 1980; Gibson et al, 1981). I.t. administration of opiates produces dose-dependent antinociception that can be blocked by pretreatment with naloxone (Yaksh & Rudy, 1978; Yaksh, 1981). The modulation of spinal antinociception has been characterized through the use of selective opiate-receptor agonists and antagonists. I.t. administration of μ-selective antagonists elicited antinociception that was blocked by β-FNA, but not naloxonazine, indicating a μ_2 mechanism of action (Paul et al., 1989). δ and κ selective agonists administered i.t. produced antinociception that was blocked by selective antagonists for these receptors, indicating that δ and κ opioid receptors are essential in mediating antinociception from spinal cord neurons (Yaksh, 1984a; Porreca et al., 1984, 1987; Wuster et al., 1980; Heyman et al., 1987, 1988). Norepinephrine and serotonin administered i.t. produces dose-dependent antinociception that is blocked by their specific receptor antagonists (Wang, 1977; Yaksh & Wilson, 1979; Reddy et al., 1980; Reddy and Yaksh, 1980; Schmauss et al., 1983; Howe et al., 1983; Fleetwood-Walker et al., 1985; Crisp et al., 1986; Kellstein et al., 1988; Castiglioni et al., 1978). The effect of norepinephrine and serotonin interactions with opioid-mediated spinal antinociception has produced conflicting results. I.t. administration of naloxone has no effect on spinal norepinephrine antinociception (Kellstein et al., 1988) and no cross-tolerance is seen between i.t. administration of morphine and ST-91, an α_2 adrenergic agonist (Tung et al., 1981). Other studies, however, have shown the development of cross-tolerance between i.t. injections of morphine and noradrenergic agonists (Solomon & Gebhart, 1987). Studies that examined at whether serotonin spinal antinociception was mediated by

opioids were mixed. I.t. injections of naloxone inhibited i.t. serotonin antinociception (Kellstein et al., 1988). However, cross-tolerance was not observed between i.t. morphine and seratonin injections (Loomis et al., 1987). Spinally-mediated opioid antinociception appears distinct yet interactive with supraspinally-mediated opioid antinociception. As noted previously, supraspinally-mediated morphine-induced antinociception is dependent upon spinal serotonergic and noradrenergic receptors yet is unaffected by spinally-administered naltrexone which blocks intrathecal morphine-induced antinociception (Yeung et al., 1980a). In contrast, supraspinally-mediated β-END-induced antinociception is blocked by spinal opioid, but not serotonergic or noradrenergic antagonist pretreatment, and supraspinal β-END administration fosters spinal met-enkephalin release (see review: Tseng, 2001). However, a classic interaction study (Yeung et al., 1980b) indicated that synergistic antinociceptive interactions occurred between ventricularly-applied and intrathecally-applied morphine. The next section briefly describes the supraspinal opioid pain-inhibitory system.

Supraspinal Systems: According to Basbaum and Fields' (1984) original three-tiered model, pain control circuits originated in the ventrolateral periaqueductal gray (vlPAG), then synapsed in the rostral ventromedial medulla (RVM), including the nucleus raphe magnus (NRM), the nucleus reticularis giganotcellularis (NRGC) and the nucleus reticularis gigantocellularis pars α, and finally projected to the substantia gelatinosa of the spinal cord through the dorsolateral funiculus (DLF) (see reviews: Fields and Basbaum, 1978; Basbaum and Fields, 1984; Bodnar, 2000; Tershner and Helmstetter, 1995). Electrical stimulation or microinjection of opiates in the vlPAG, generate antinociception by ultimately inhibiting neurons in the spinal cord. Opiate

agonists administered in the RVM also block nociceptive responses. (Bodnar et al., 1988; Jensen et al., 1986; Satoh et al., 1983). Evidence suggests that there is a direct projection between the vlPAG and various nuclei in the RVM. Lesions or injection of local anesthetic in the RVM or the DLF block the effects of midbrain stimulation (Prieto et al., 1983; Sandkuhler et al., 1982; Basbaum and Fields, 1984).

In addition to voluminous work studying the nature of opioid-induced antinociception in the brainstem sites, several studies have shown that changes in nociception in response to stress often depends on neurons within the amygdaloid complex (Helmstetter et al., 1992; Helmstetter et al., 1993). Microinjection of opiates into the amygdala produce profound antinociception (Helmstetter et al., 1993; Rodgers, 1977). Presumably, the opioid-mediated antinociception elicited from the amygdala is achieved via connections with the vlPAG (Beitz, 1982; Krettak & Price, 1978). Thus, it appears that there are several supraspinal structures that are linked anatomically and physiologically, and play a crucial role in the mediation of opioid-mediated antinociception (see review: Bodnar, 2000).

Two approaches have been used to identify the neurochemical substrates involved in opioid-mediated antinociception between two sites within this proposed descending pain-control pathway. The first approach determines whether opioid antinociception elicited from one site can be blocked by pretreatment with an antagonist in a second site. This antagonist approach addresses several issues. First, full dose-response curves of antagonigsts are studied to see whether the antagonist alone has any effects on basal nociceptive responses (e.g., hyperalgesia). Further, control placements, either dorsal or lateral to the site of interest, are used to assess the site specificity of the antagonist

effects. The second approach studying the relationship between sites involved in the descending pain pathway examine synergistic effects between two sites. Synergy is a greater than additive effect when sub-threshold doses of opioid agonists are administered simultaneously in two different sites (see review: Bodnar, 2000).

First Approach: Using the two criteria listed above, relationships were established between the vlPAG and the RVM, and the amygdala and vlPAG for opioid agonists. The direct projections between the vlPAG and the RVM appear to contain serotonin fibers (Abols & Basbaum, 1981; Beitz, 1982; Beitz et al., 1983). Autoradiography confirmed the presence of serotonin receptors on RVM neurons (Pazos et al., 1985; Waeber, 1988). Pretreatment of the RVM with either general (methysergide), 5HT2 (ritanserin) or 5HT3 (ICS205930) receptor antagonists significantly and dose-dependently inhibited morphine antinociception elicited from the vlPAG. These antagonist effects appeared to be selective to opioid antinociception since antagonists alone failed to produce changes in nociceptive responses. The effects also seem to be site-specific, since cannulae placed dorsal or lateral to the RVM failed to alter vlPAG antinociception (Kiefel et al., 1992a; Keifel et al., 1992b). It has also been established that the projections between the vlPAG and RVM also contain enkephalin fibers (Abols & Basbaum, 1981; Beitz, 1983). Pretreatment in the RVM with either general (naltrexone), μ (β-FNA) or δ (naltrindole)-opioid receptor antagonists significantly and dose-dependently inhibited morphine antinociception elicited from the vlPAG (Kiefel et al., 1993). The antagonist effects were selective and site-specific (Bodnar et al., 1988). Excitatory amino acid (EAA) receptors, especially the NMDA receptor, have been implicated in mediating supraspinal antinociceptive effects.

Pretreatment of the RVM with either competitive NMDA (AP-7), noncompetitive NMDA (MK-801) or kainate/AMPA (CNQX) EAA receptor antagonists showed differential results. Both of the NMDA receptor antagonists significantly and dose-dependently altered morphine antinociception elicited from the vlPAG; however, the kainate/AMPA receptor failed to do so. The effects of the EAA antagonists were selective and site specific (Spinella et al., 1996). Cholinergic drugs administered into medullary sites produce antinociception (Brodie & Proudfit, 1984; Iwamoto, 1989; Klamt & Prado, 1991). Pretreatment of RVM with either muscarinic (scopolamine), M_1 (pirenzepine), M_2 (methoctramine) or nicotinic (mecamylamine) cholinergic receptor antagonists resulted in significant and dose-dependent inhibition of morphine antinociception elicited from the vlPAG. Although these effects were selective, however, they were not site-specific since cannulae placed dorsal and lateral to the meduallary site of interest were also effective in blocking vlPAG antinociception (Spinella et al., 1997).

The functional relationship between the amygdala and the vlPAG has also been established by examining at opioid-opioid interactions. Pretreatment of the vlPAG with either general (naltrexone), or δ_2 (naltrindole isothiocyanate) opioid receptor antagonists resulted in an inhibition in morphine antinociception elicited from the amygdala, but not following pretreatment with the μ (β-FNA) receptor antagonist in the vlPAG (Pavlovic et al., 1996). Similar results were seen when β-endorphin antinociception was assessed in the amygdala. Pretreatment of the vlPAG with either general or δ_2, but not μ, receptor antagonists inhibited β-endorphin antinociception elicited from the amygdala (Pavlovic et al., 1996). U50 488H-induced antinociception elicited from the amygdala was blocked by either general, μ- or δ_2-opioid receptor antagonists administered into the vlPAG

(Pavlovic and Bodnar, 1998b). Finally, DAMGO-induced antinociception elicited from the amygdala was blocked by pretreatment with either general or μ-antagonists, but not β-endorphin$_{1-27}$ (an antagonist for the putative ε receptor) in the vlPAG (Tershner and Helmstetter, 2000).

Second Approach: The second approach used to study site interactions used at synergy studies. Synergy is defined as a greater than additive effect when sub-threshold doses of opioid agonists are used in two different sites (see review: Bodnar, 2000). Synergy studies have been used to establish relationships between both the vlPAG and RVM, and the amygdala and vlPAG. Subthreshold doses of morphine, which are inactive when administered alone, produced a potent antinociceptive response when simultaneously coadministered in the vlPAG and RVM (Bodnar et al., 1991; Rossi et al., 1993, 1994). Synergistic interactions between the vlPAG and RVM were also observed when subthreshold doses of the μ-receptor agonist, DAMGO, the δ_2-receptor agonist deltorphin II, and a combination of DAMGO and deltorphin II were administered into each site (Sutters et al., 1990, Heyman et al., 1998; Rossi et al., 1994). Synergistic antinociceptive interactions have also been examined between the amygdala and the vlPAG using morphine and β-endorphin (Pavlovic & Bodnar, 1998). Simultaneous and subthreshold doses of morphine in both sites and β-endorphin in both sites produced synergistic interactions. When morphine and β-endorphin were given in combination, synergistic interactions were seen only when morphine was administered in the amygdala and β-endorphin in the vlPAG, but not vice versa (Pavlovic & Bodnar, 1998). Thus, these approaches indicate that the amygdala has functional similarities to the other pain-

inhibitory nuclei; the next sections detail the role of this nucleus in opioid-mediated and stress-mediated antinociception.

I. The Role of the Amygdala in Opioid-induced Antinociception:

Several forebrain regions, especially the amygdala, have been implicated to be a part of this endogenous pain-inhibitory pathway (Helmstetter et al., 1993; Manning and Mayer, 1995a; 1995b; Pavlovic et al., 1996). Stimulation of the amygdala inhibits the activity of dorsal horn spinothalamic neurons (Carstens, 1986). Systemically administered morphine requires the integrity of the amygdala in order to suppress spinal nociceptive signals (Mayer and Manning, 1995b). Bilateral lesions placed in the amygdala (including the central (Ce), and basolateral (BLA) nuclei) attenuate morphine-induced antinociception on several nociceptive assays (Helmstetter et al., 1993; Mayer and Manning, 1995a; 1995b; Pavlovic et al., 1996). Microinjection of either μ-opioid agonists (morphine and DAMGO), β-endorphin, an enkephalinase inhibitor, neurotensin or carbachol into the amygdala result in antinociception on the tail flick test (Kalivas et al., 1982; Helmstetter et al., 1993; Manning and Mayer, 1995a; 1995b; Pavlovic et al., 1996; Al-Rodhan et al., 1990; Klamt and Prado, 1991; Helmstetter et al., 1993, 1995; Pavlovic et al., 1996). Morphine, β-endorphin, and the selective κ-receptor agonist, U50 488H produced antinociception on the jump test (a test measuring reactivity to shock) following amygdala microinjections (Rodgers, 1977, 1978; Pavlovic et al., 1996; Pavlovic and Bodnar, 1998a). Neither the κ-selective agonist, U50 488H, or the δ-selective opioid agonist, DPDPE, were capable of eliciting antinociception from the amygdala on the tail-flick test (Helmstetter et al., 1995; Pavlovic and Bodnar, 1998b).

Studies have shown that there are anatomical and functional relationships between the amygdala and other nuclei involved in the descending pain-inhibitory pathway, especially that of the vlPAG. There are direct reciprocal connections project between the amygdala and vlPAG (Tershner and Helmstetter, 1995; Pavlovic et al., 1996; Helmstetter et al., 1998). As detailed previously, an opioid synapse in the PAG appears to be essential for the full expression of antinociception elicited by amygdala microinjections. Pretreatment of the PAG with general or selective (particularly δ) opioid-receptor antagonists results in inhibition of opioid agonist-induced antinocicption elicited from the amygdala (Pavlovic 1996a, 1996b, 1998; Helmstetter et al., 1998).

J. The Role of the Amygdala in Stress:

The amygdala has been implicated involved in the regulation of the neuroendocrine stress response. The paraventricular nucleus (PVN) of the hypothalamus, especially the corticotropin releasing factor-(CRF)-containing neurons, controls the stress-response (Bohus et al., 1996). There are some direct connections between the hypothalamic PVN and the amygdala (Silverman et al., 1981; Tribollet and Dreifuss, 1981; Gray et al., 1989; Marcilhac and Siaud, 1996); however, the amygdala also has indirect connections to the PVN via the bed nucleus of the stria terminalis (BNST) (Krettek and Price, 1978; Weller and Smith, 1982; DeOlmos et al., 1985). The amygdala also contains CRF-containing neurons with widespread projections throughout the neuroaxis (Gray and Bingaman, 1996). Lesions of the PVN CRF-containing neurons increases CRF expression in the central nucleus (Ce) of the amygdala, indicating that compensatory mechanisms in the amygdala can replace the actions of PVN neurons

(Walker et al., 1997). Lesions placed in the Ce and BLA nuclei of the amygdala blocked conditioned increases in corticosterone release (Goldstein et al., 1996).

The amygdala also plays a modulatory role in the stress response by regulating the release of dopamine (DA), serotonin (5-HT) and norepinephrine (NE) in the prefrontal cortex (PFC) as a result of exposure to mild stressors (Goldstein et al., 1996). Lesions in the amygdala block the stress-induced increases in DA release in the frontal cortex following mild footshock or exposure to a novel environment (Davis et al., 1994).

The amygdala also appears to be important in the mediation of some, but not all forms of opioid-mediated stress-induced analgesia. Thus, forms of stress –induced analgesia (e.g., conditioned fear, exposure to shock, exposure to a predator) that can be blocked by general opioid antagonist pretreatment, are also reduced following lesions placed in the amygdala (Helmstetter, 1992; 1993; Helmstetter and Bellgowan, 1993; Fox and Sorenson, 1994; Bellgowan and Helmstetter, 1996; but see Watkins et al., 1993). In contrast, lesions placed in the amygdala failed to affect analgesia elicited by either continuous (nonopioid-mediated) or intermittent (opioid-indicated) cold-water swims (Pavlovic et al., 1996b). Given the putative role of OFQ/N in stress-related responses (Goldstein et al., 1996; Walker et al., 1997), the amygdala would appear to be an ideal site to study such relationships. This is considered in the next Rationale section.

K. Rationale:

To recapitulate, during the successful cloning of the traditional types of opioid receptor (μ, κ, δ) (Evans et al., 1992; Kieffer et al., 1992), a fourth orphan-like opioid receptor was identified, the ORL-1 receptor, which did not bind traditional opioid agonists with high affinity (Mollereau et al., 1994). Subsequently, an endogenous ligand

for the ORL-1 receptor was identified, rat-pre-proOrphanin FQ/Nociceptin$_{135-151}$ (rppOFQ/N$_{135-151}$) (Meunier et al., 1995; Reinscheid et al., 1995) which is part of a prepro-Orphanin (ppOFQ) precursor gene (see reviews: Meunier, 1997, 2000). Initial studies using rppOFQ/N$_{135-151}$ demonstrated that this ligand had hyperalgesic, anti-stress and/or anti-opioid properties following ventricular administration in rodents (Meunier et al., 1995; Reinscheid et al., 1995; Mogil et al., 1996a, 1996b; Tian et al., 1997). However, subsequent studies found that rppOFQ/N$_{135-151}$, and two of its truncated fragments, rppOFQ/N$_{135-145}$ and rppOFQ/N$_{135-14}$·, as well as other related peptides from the ppOFQ/N precursor gene (e.g., rppOFQ/N$_{154-181}$), display dose-dependent, route-dependent and time-dependent antinociception (Rossi et al., 1996, 1997, 1998; King et al., 1997; Tian et al., 1997; Jhamandas et al., 1998; Shane et al., 2000), and that pain control nuclei such as the vlPAG, the LC and the RVM differentially elicit these antinociceptive effects (Rossi et al., 2002).

The nuclei of the amygdala are also known to elicit antinociceptive responses following administration of μ-receptor agonists morphine or DAMGO, β-endorphin, or the κ-selective agonist U50 488H, which depend on an opioid synapse in the vlPAG for their full expression (Pavlovic et al., 1996a, 1996b, 1998; Helmstetter et al., 1998; Tershner and Helmstetter, 2000). Thus, pretreatment of the vlPAG with general and selective opioid receptor antagonists results in reduced opioid agonist-induced antinociception elicited from the amygdala. In addition, the autoradiographic studies have shown that the amygdala has an abundance of ORL-1 receptors (Letchworth et al., 2000) as well as other opioid receptor subtypes (Mansour, 1987). Since the amygdala is also intimately involved in the mediation of stress-related responses (Goldstein et al.,

1996; Walker et al., 1997), this makes it an ideal candidate for mediating the multi-faceted actions of rppOFQ/$N_{135-151}$.

Thus, the purpose of the present dissertation is to characterize the novel "orphan" ORL-1 receptor and its endogenous ligand, OFQ/N through analysis of pharmacological, anatomical, and mostly behavioral evidence. Four series of specific experiments are proposed.

Specific Aim One evaluated the analgesic and hyperalgesic effects of rppOFQ/$N_{135-151}$ and two of its truncated fragments rppOFQ/$N_{135-145}$ and rppOFQ/$N_{135-141}$ in the amygdala in rats on both radiant heat (tail-flick) and shock (jump threshold) tests. These fragments were chosen because rppOFQ/$N_{135-151}$ and its two active truncated versions, rppOFQ/$N_{135-145}$ and rppOFQ/$N_{135-141}$ were found to produce antinociception in mice following ventricular administration, which was blocked by antisense probes targeted at exons 2 and 3, but not exon 1 of the ORL-1 clone (Rossi et al., 1997). Two different nociceptive tests were employed to allow for the assessment of generalizability vs. specificity across nociceptive stimuli. Although the vlPAG is a site at which traditional opioid agonists like morphine and β-endorphin produce marked antinociception on both tests (e.g., Kiefel et al., 1992a; 1992b; 1993; Spinella et al., 1996; 1997; 1999), the amygdala is a site at which traditional opioid agonists like morphine, β-endorphin and U50 488H produce marked antinociception on the jump (a more reactive measure) than on the tail-flick (a more reflexive measure) test (Pavlovic et al., 1996; Pavlovic and Bodnar, 1998). The tail-flick test is an ideal measure with which to assess both antinociceptive and hyperalgesic responses within the same paradigm such that high-intensity radiant heat elicits short latencies that are conducive to measurement of

antinociception (observing increases in latencies) and lower-intensity radiant heat elicits longer latencies that are conducive to measurement of hyperalgesia (observing decreases in latencies). rppOFQ/$N_{135-151}$ and its fragments were tested over a very wide dose range since this peptide can conceivably produce an inverted U-shaped function rather than the traditional linear dose-response relationships for antinociception elicited by μ opioid antagonists. The studies included in this specific aim have been published (Shane et al., 2001) in the journal, Brain Research.

Specific Aim Two evaluated the antinociceptive and hyperalgesic effects of a related peptide, rppOFQ/$N_{154-181}$ in the amygdala of rats on both radiant heat and shock tests according to a similar strategy to that employed in the first study. This peptide is at the end of the pre-proOFQ gene. Initial studies with this and other related peptides found that it elicited similar antinociceptive effects to rppOFQ/$N_{135-151}$ when given i.c.v. in mice (Rossi et al., 1998). The present study examined rppOFQ/$N_{154-181}$ in the amygdala, and was part of a larger study evaluating the effects of this peptide in other supraspinal sites as well including the vlPAG, RVM and LC. The studies included in this specific aim have been published (Rossi et al., 2002) in the Journal of Pharmacology and Experimental Therapeutics.

Specific Aim Three determined whether rppOFQ/$N_{135-151}$ antinociception in the amygdala can be blocked by pretreatment in the same amygdala site with equimolar doses of either general (naltrexone), μ (β-FNA), κ (NBNI) or δ (NTI) opioid receptor subtypes in rats. Autoradiographic and immunihistochemical techniques have shown that there is a dense distribution of ORL-1 and traditional opioid receptors in the amygdala (Letchworth et al., 2000; Mansour et al., 1987, 1994a, 1994b, 1994c, 1995a, 1995b,

1996). Antinociception elicited following amygdala microinjections of the selective κ_1 agonists, U50 488H, is blocked by amygdala pretreatment of general and selective κ_1 opioid antagonists. This present studies purpose was to elucidate whether rppOFQ/$N_{135-151}$–induced antinociception is mediated through a functional circuit utilizing the traditional opiate receptors. The studies included in this specific aim have been accepted for publication (Shane et al., 2002) in the journal, Analgesia.

Antinociceptive responses following amygdala microinjections of traditional opioid agonists such as morphine, DAMGO, β-endorphin and U50 488H have been linked to an intrinsic pain-inhibitory pathway originating in the vlPAG. (Krettak and Price, 1978; Beitz, 1982; Basbaum and Fields, 1984; Rizvy et al., 1991). Pretreatment of the vlPAG with lidocaine or microinjections of either general or selective μ or δ_2 opioid receptor antagonists significantly reduced the antinociception elicited by either morphine, β-endorphin, DAMGO or U50 488H in the amygdala (Pavlovic et al., 1996; Pavlovic et al., 1998; Helmstetter et al., 1998; Tershner and Helmstetter, 2000). **Specific Aim Four** evaluated whether rppOFQ/$N_{135-151}$ antinociception elicited from the amygdala can be blocked by pretreatment with equimolar doses of general (naltrexone), μ (β-FNA), κ (NBNI) and δ (NTI) opioid receptor subtypes in the vlPAG in rats to indicate whether this peptide utilizes a similar anatomical and functional pathway to that of the traditional opioid agonists. Site-specificity of any positive antagonist effects within the vlPAG was assessed by determining antagonist effectiveness in misplaced mesencephalic placements. Finally, reciprocal ascending influences were examined, the ability of selective opioid antagonists administered into the amygdala were assessed for effects upon rppOFQ/$N_{135-151}$-induced antinociception elicited from the vlPAG.

CHAPTER 2. GENERAL METHODS

Subjects, Surgery and Histology: Adult male albino Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, 80-100 days of age) were housed individually in polyethylene cages and maintained on a 12:12 h light:dark cycle with food and water available ad libitum. Following anesthetization with chlorpromazine HCL (3 mg/kg, i.p.) and ketamine HCl (120 mg/kg, i.m.), a stainless-steel guide cannula (26-gauge, Plastics One, Roanoke, VA) was placed stereotaxically (Kopf Instruments, Tujunda, CA) into the amygdala in all experiments, the amygdala and either the vlPAG or a control misplaced mesencephalic site lateral to the vlPAG in experiment four, and bilaterally into the amygdala and the vlPAG in experiment four. Stereotaxic coordinates were: (a) unilateral amygdala: incisor bar (-3.3mm), 2.8 mm posterior to the bregma suture, 3 mm lateral to and angled 13° away from the sagittal suture, and 8.4 mm from the top of the skull (Experiments 1, 2, 3 and 4), (b) bilateral amygdala: incisor bar (-3.3 mm), 2.8 mm posterior to bregma suture, 4.5 mm lateral to and angled 5° away from the sagittal suture, and 8.4 mm from the top of the skull (Experiment 4), (c) vlPAG: incisor bar (-5 mm), 0.5 mm anterior to the lambda suture, 1.5 mm lateral to and angled 12° toward the sagittal suture, and 6.8 mm from the top of the skull (Experiment 4), and (d) misplaced control: incisor bar (-5 mm), 0.5 mm anterior to the lambda suture, 2.0 mm lateral to the sagittal suture, and 6.8-7.0 mm from the top of the skull (Experiment 4). Cannulae were secured to three anchor screws with dental acrylic. All animals were allowed one week to recover from surgery and clear anesthetic. After the completion of testing, all animals were anesthetized (Euthanasia, Henry Schein), and received a transcardiac perfusion with 0.9% normal saline followed by 10% buffered formalin. The

brains were removed, blocked and cut coronally in 40 μm sections through the cannula placement. The tissue, stained with cresyl violet was examined using light microscopy by an observer unfamiliar with the behavioral data. Only animals with confirmed cannulae placements were included in the data analysis.

Nociceptive tests: A tail-flick analgesiometer (ITTC, Woodland Hills, CA) provided a radiant heat source that was mounted 8 cm above the rat's tail. Radiant heat was applied 3-9 cm proximal to the tip of the rat's tail: removal of the tail activated a photocell and determined the latency (0.01-s accuracy). In the first nociceptive paradigm, a high-intensity thermal stimulus was set to produce baseline tail-flick latencies between 2 and 3.5 s. Each session consisted of 3 latency determinations at different points on the tail at 10 –s intervals. To avoid tissue damage, a trial was automatically terminated if a response did not occur within 12 s. Baseline latencies were determined for at least 4 days before experimental testing began to insure stability of responding. This paradigm was used to assess antinociception (Experiments 1, 2, 3 and 4). In the second nociceptive paradigm, radiant heat was applied in an identical manner to that described above except that a lower-intensity thermal stimulus was applied to produce baseline tail-flick latencies between 7 and 10 s with an automatic cut-off of 20 s. This paradigm was capable of assessing both antinociception and hyperalgesia (pronociception) (Experiments 1 and 2).

A third nociceptive paradigm was employed to examine reactivity to shock (jump test). Rats were tested on this measure in Experiments 1 and 2 immediately after the high-intensity tail-flick testing. A previous study has demonstrated that there is no carry-over effect when the tail-flick test is done prior to the jump test; however, the reverse is not

true (Kelly et al., 1982). Electric shock was delivered to the feet of the rat by a shock generator (BRS/LVE) and a shock scrambler (Campden Instruments). The jump threshold was defined in mA as the lowest of two consecutive ascending intensities in which the animal simultaneously removed both hindpaws from the grids. Each of the six trials began with the animal receiving a 300-ms footshock at a current intensity of 0.10 mA with subsequent shocks increased in 0.05 mA steps at 10-s intervals until either the jump threshold was determined or a cutt-off threshold of 1.2 mA was reached. Baseline thresholds were determined for at least 4 days before experimental testing began to ensure stability of responding. All animals displayed consistent latencies and thresholds in baseline and vehicle testing that did not appear subject to desensitization.

Peptides, Antagonists and Microinjections: rppOFQ/$N_{135-151}$ was synthesized by Dr. G.W. Pasternak (Experiment 1) or purchased from Peninsula Laboratories (Belmont, CA, Experiments 3 and 4). rppOFQ/$N_{135-145}$ and rppOFQ/$N_{135-141}$ were synthesized by Dr. G.W. Pasternak. (Experiment 1). rppOFQ/$N_{154-181}$ was synthesized by Dr. R.G. Allen (Experiment 2). Naloxone (Experiment 2) and naltrexone (NTX: Experiment 3 and 4) were purchased from Sigma Chemical Co. (St. Louis, MO). β-FNA, NBNI and NTI were all purchased from Sigma RBI and used in Experiments 3 and 4. All peptides and antagonists were dissolved fresh in 0.9% saline, and were microinjected at 3-8 h into the light cycle in 0.1 µl volumes at a rate of 0.2 µl every 10 s through a stainless-steel internal cannula (33-gauge, Plastics One) which was connected to a Hamilton microsyringe by polyethylene tubing. A dummy cannula was in place before and after microinjections to insure patency of the guide cannula. Injection treatments were separated by at least 1 week to minimize tolerance. rppOFQ/$N_{135-151}$, rppOFQ/N_{135-}

$_{145}$, rppOFQ/N$_{135-141}$ and rppOFQ/N$_{154-181}$ were made daily immediately before testing. Microinjections of either NTX (20 min), β-FNA (16-23 h), NBNI (1 h) or NTI (30 min) preceded agonist microinjections to reflect the peak opioid antagonist activity (Mattia et al., 1992; Portoghese et al., 1980, 1987, 1990; Takemori et al., 1981; Zukin and Zukin, 1981).

Statistical Analyses: Two-way repeated measures analyses of variance or randomized block analyses of variance assessed significant effects, with experimental conditions as one variable and test times as the second variable, and the interaction between conditionsand times. Tukey controlled comparisons (p, 0.05) were used to determine the antinociceptive effects of OFQ/N across conditions relative to corresponding vehicle treatment, and/or antagonist actions.

CHAPTER 3: SPECIFIC AIM ONE

rppOFQ/$N_{135-151}$, rppOFQ/$N_{135-145}$ and rppOFQ/$N_{135-141}$ Antinociception and Hyperalgesia Elicited from the Amygdala:

Introduction

The present study examined the effects of equimolar doses of either rppOFQ/$N_{135-151}$, rppOFQ/$N_{135-145}$ or rppOFQ/$N_{135-141}$ administered into the amygdala would produce antinociception on the tail-flick test using high intensity thermal stimuli to elicit short baseline latencies as well as on the jump test. It should be noted that morphine, β-endorphin and U50 488H antinociception elicited from the amygdala is more robust on the jump test relative to the tail-flick test (Pavlovic and Bodnar, 1998a; Pavlovic et al., 1996a; Rodgers, 1977, 1978). Hyperalgesia cannot be typically observed in traditional tail-flick paradigms due to short baseline latencies. Therefore, as performed previously (Rossi et al., 1996), the present study evaluated potential hyperalgesic effects of rppOFQ/$N_{135-151}$, rppOFQ/$N_{135-145}$ and rppOFQ/$N_{135-141}$ elicited from the amygdala by decreasing the intensity of the thermal stimulus, lengthening baseline latencies and permitting hyperalgesia to be potentially detected.

Methods

A total of seven different protocols were employed and are summarized on Table 1. The first three protocols employed the high-intensity tail-flick test to assess: a) a dose-response curve (0.027-54.27 μg) for rppOFQ/$N_{135-151}$-induced antinociception in the amygdala; b) a dose-response curve (1.65-33.0 μg) for rppOFQ/$N_{135-145}$-induced antinociception in the amygdala; and c) a dose-response curve (0.1-19.6 μg) for

Table 1. Summary of experimental groups in Experiment 1.

A. rppOFQ/$N_{135-151}$-induced antinociception on high-intensity tail flick test:

µg	nmol	n
Vehicle		54
0.027	0.015	7
0.27	0.15	6
1.5	0.75	8
2.7	1.5	6
5.5	3	6
10	5.5	8
20	11	7
27.14	15	6
54.27	30	6

B. rppOFQ/$N_{135-145}$-induced antinociception on high-intensity tail flick test:

Vehicle		24
1.65	1.5	6
3.3	3	6
6.04	5.5	6
12.08	11	4
16.5	15	7
33	30	7

C. rppOFQ/$N_{135-141}$-induced antinociception on high-intensity tail flick test:

Vehicle		7
0.1	0.15	6
1.0	1.5	6
7.23	11	6
19.6	30	6

D. Low-intensity tail flick test (hyperalgesia):

	Vehicle		8
rppOFQ/$N_{135-151}$	20	11	8
rppOFQ/$N_{135-145}$	12.14	11	8
rppOFQ/$N_{135-141}$	7.23	11	8

E. rppOFQ/$N_{135-151}$-induced antinociception on jump test:

Vehicle		15
10	5.5	8
20	11	7

F. rppOFQ/$N_{135-145}$-induced antinociception on jump test:

Vehicle		15
16.5	15	7
33	30	7

G. rppOFQ/$N_{135-141}$-induced antinociception on jump test:

Vehicle		15
7.2	11	7
19.6	30	7

rppOFQ/$N_{135-141}$-induced antinociception in the amygdala. The fourth protocol employed the low-intensity tail-flick test such that one dose of each OFQ/N fragment was injected into the amygdala and all animals were tested for hyperalgesia: rppOFQ/$N_{135-151}$ 20μg, rppOFQ/$N_{135-145}$ 12.14μg, and rppOFQ/$N_{135-141}$ 11.06μg). The last three protocols employed the jump test for a dose-response curve (5.5-11.0 μg) for OFQ/N-induced antinociception in the amygdala; a dose-response curve (15.0-30.0 μg) for rppOFQ/$N_{135-145}$-induced antinociception in the amygdala; and a dose-response curve (11.0-30.0 μg) for rppOFQ/$N_{135-141}$-induced antinociception in the amygdala. In all protocols, tail-flick latencies and jump thresholds were determined 5, 15, 30 and 60 minutes following vehicle treatment and agonist microinjections. There was a one-week interval between particular conditions; this interval minimized agonist-induced tolerance effects. No animal in any protocol received more that five microinjection conditions in a given site.

Results

Histological verification. The 121 rats in the treatment protocols had cannula placements in the central and baso-lateral nuclei of the amygdala. The placements of the cannulae across paradigms were quite similar, and no significant differences were observed in the magnitude of OFQ/N-induced antinociception between nuclei, and thus data for each agonist treatment for the two amygdala placements were pooled.

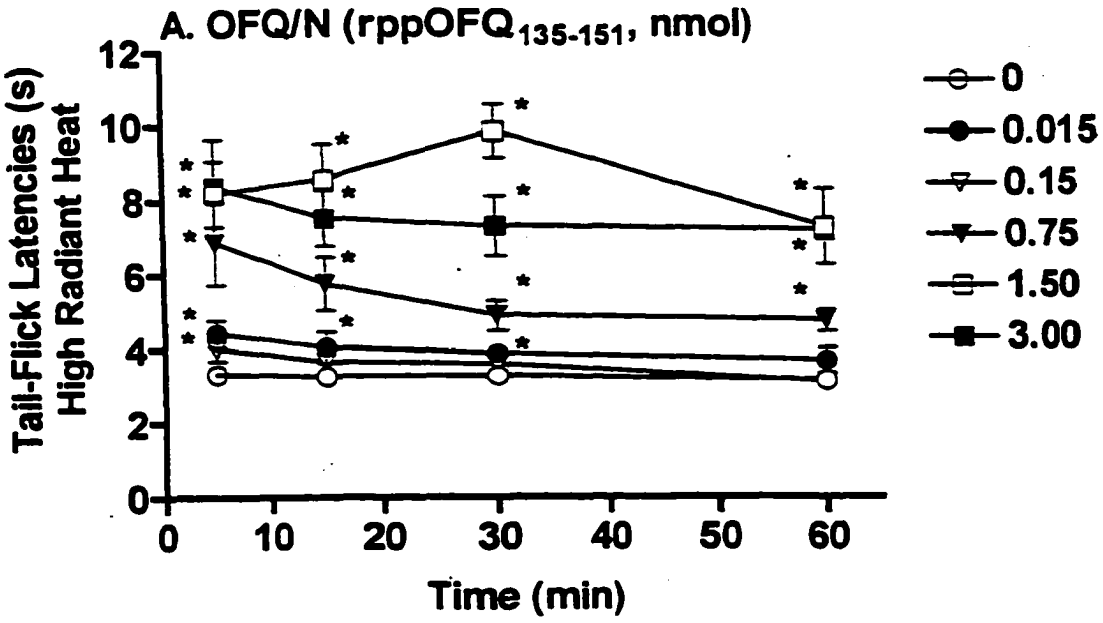
Amygdala rppOFQ/$N_{135-151}$ and the high-intensity tail-flick assay. Significant differences were observed among experimental conditions ($F(9,477)=1389.43$, $P<0.001$), across test times ($F(3,159)=607.34$, $P<0.001$) and for the interaction between conditions and times ($F(27,1431)=81.68$, $P<0.001$). A wide range (0.015-30 nmol) of rppOFQ/$N_{135-151}$ doses in the amygdala significantly increased high-intensity tail-flick latencies with

effects observed up to 5 min following the 0.15-nmol dose, up to 15 min following the 11nmol dose, up to 30 min following the 0.015-nmol dose and across the 60-min time course following the 0.75-5.5- and the 15-30 –nmol dose ranges (Fig. 2). The magnitude of rppOFQ/$N_{135-151}$-induced antinociceptive effects in the amygdala displayed an inverted U-shaped function such that latencies increased from 0.15 to 0.75 nmol, peaked at 1.5-3 nmol, and then declined in analgesic efficacy from 5.5 to 30 nmol.

Amygdala rppOFQ/$N_{135-145}$ and high intensity tail-flick assay. Significant differences were observed among conditions ($F(6, 138)=68.55$, $P<0.001$), across times ($F(3, 69)=158.42$, $P<0.001$) and for the interaction between conditions and times ($F(18, 414)=34.68$, $P<0.001$). Whereas low (1.5-5.5 nmol) doses of rppOFQ/$N_{135-145}$ produced meager and transient effects upon high-intensity tail-flick latencies in the amygdala, latencies significantly increased for up to 15 min following the 11-nmol dose and across the time course following the 15- and 30-nmol doses (Fig. 3). The greatest magnitude of rppOFQ/$N_{135-145}$-induced antinociception in the amygdala occurred 5 min following the 15-nmol dose, an effect (5.4 s) that was considerably less than that observed for the larger OFQ/N (8-9.4 s).

Amygdala rppOFQ/$N_{135-141}$ and the high-intensity tail-flick assay. Significant differences were observed among conditions ($F(4,24)=7.06$, $P<0.007$). across times ($F(3,18)=7.30$, $P<0.021$) and for the interaction between conditions and times ($F(12,72)=3.32$, $P<0.007$). Whereas low doses of rppOFQ/$N_{135-141}$ in the amygdala produced transient, significant increases in high-intensity tail-flick latencies following the 0.15- (5 min) and 1.5- (5-15 min) nmol doses, higher (11-30 nmol) doses of rppOFQ/$N_{135-141}$ in the amygdala failed to alter latencies (Fig. 3). The greatest magnitude

Figure 2. Alterations in high-intensity tail-flick latencies (s, ± S.E.M.) following administration of rppOFQ/$N_{135-151}$ into the amygdala across a wide dose range (0.015-3 nmol, upper panel; 5.5-30 nmol, lower panel) relative to vehicle treatment. The asteriks in this and subsequent figures indicate significant increases in latencies relative to corresponding vehicle control conditions.



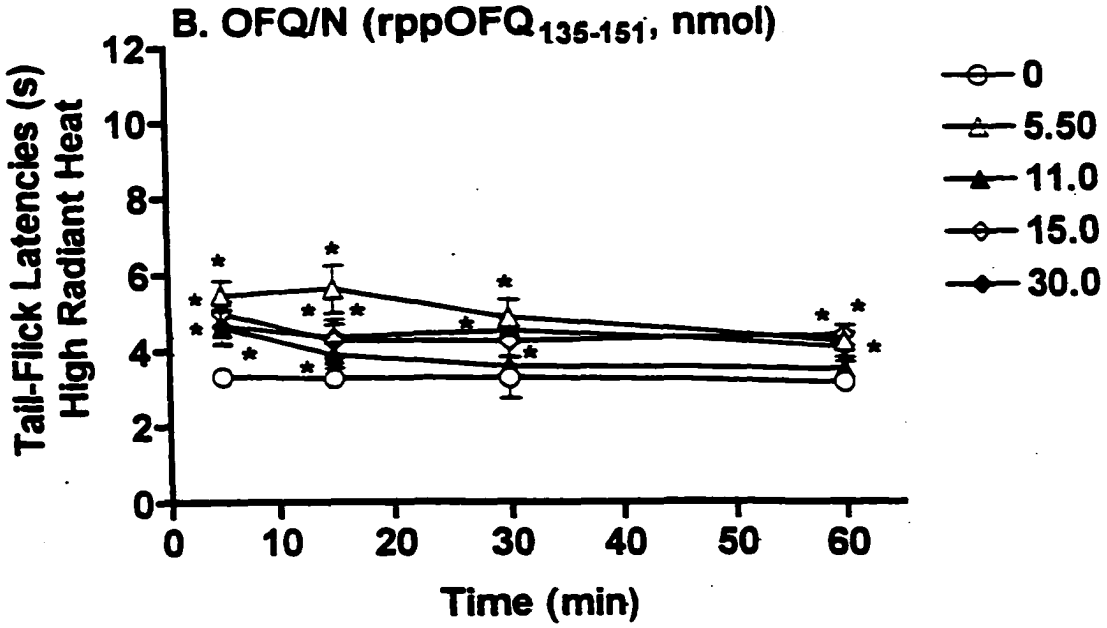
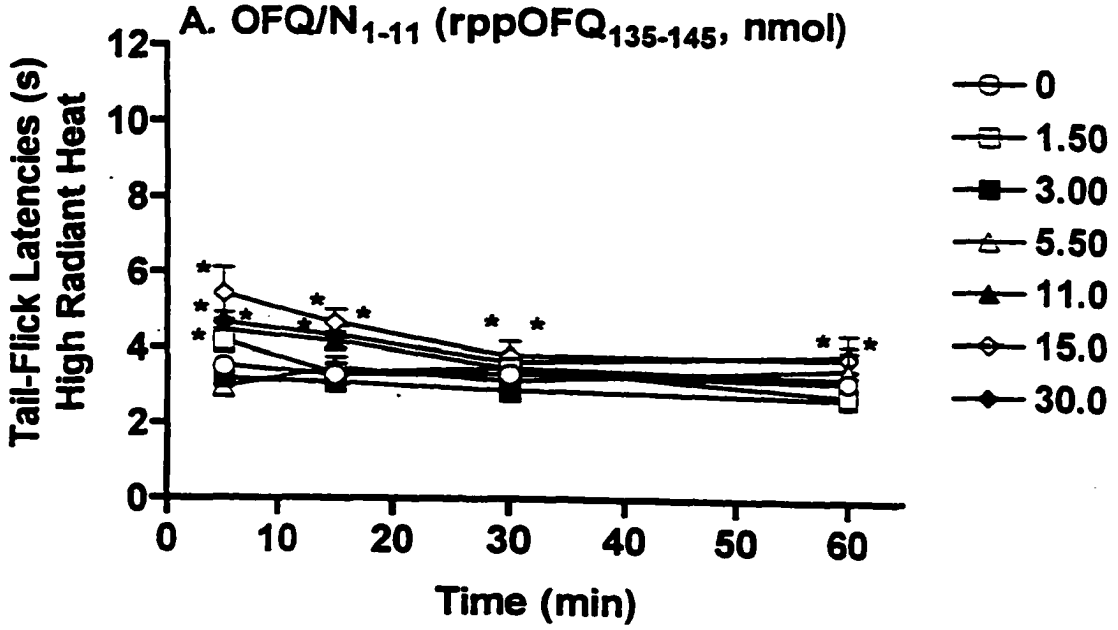
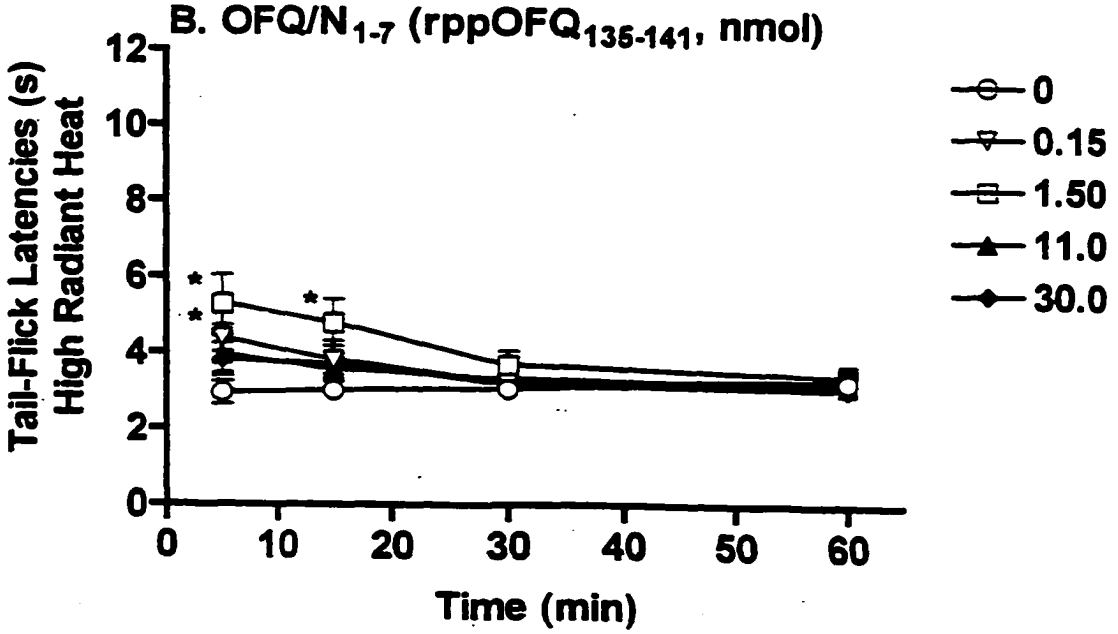


Figure 3. **Alterations in high-intensity tail-flick latencies (s, ±S.E.M.) following administration of either rppOFQ/$N_{135-145}$ (upper panel) or rppOFQ/$N_{135-141}$ (lower panel) into the amygdala relative to vehicle treatment.**





of rppOFQ/$N_{135-141}$-induced analgesia in the amygdala occurred 5 min following the 1.5-nmol dose, an effect (5.3 s) that was considerably less than that observed for the larger OFQ/N (8-9.4 s).

Amygdala OFQ/N fragments and the low-intensity tail-flick assay. Significant differences were observed among conditions ($F(3, 21)=10,25$, $P<0.002$) and for the interaction between conditions and times ($F(9,42)=2.11$, $P<0.42$), but not across times ($F(3,21)=1.87$, NS). Low-intensity tail-flick latencies failed to be altered by an equimolar (11 nmol) dose of either rppOFQ/$N_{135-151}$ or rppOFQ/$N_{135-141}$ in the amygdala, and were only transiently (15 min) but significantly increased by an equimolar dose of rppOFQ/$N_{135-145}$ in the amygdala (Fig. 4). In all cases, amygdala microinjections of each of the OFQ/N peptide fragments tended to increase low-intensity latencies rather than produce decreases in these latencies as expected for the presence of a hyperalgesic state.

Amygdala OFQ/N fragments and jump thresholds. Significant differences in the rppOFQ/$N_{135-151}$ paradigm were observed across test times ($F(3,42)=12.72$, $P<0.001$) and for the interaction between conditions and times ($F(6,84)=10.45$, $P<0.001$), but not among conditions ($F(2,28)=1.28$, NS). Jump thresholds failed to be altered by rppOFQ/$N_{135-151}$ in the amygdala following the 11-mol dose, and were only transiently (60 min) but significantly increased following the 5.5-nmol dose (Fig. 5). Significant differences in the rppOFQ/$N_{135-145}$ paradigm failed to be observed among conditions ($F(2,12)=3.62$, NS) across times ($F(3,18)=1.57$, NS) or for the interaction between conditions and times ($F(6,36)=0.54$, NS). Jump thresholds failed to be altered by either the 15- or the 30-nmol doses of rppOFQ/$N_{135-145}$ in the amygdala (Fig. 5). Significant differences in the rppOFQ/$N_{135-141}$ paradigm were obtained across times ($F(2,10)=3.20$,

Figure 4. Alterations in low-intensity tail-flick latencies (s, ±S.E.M.) following administration of either rppOFQ/$N_{135-151}$, (upper panel) rppOFQ/$N_{135-145}$ (middle panel) or rppOFQ/$N_{135-141}$ (lower panel) into the amygdala relative to vehicle treatment.

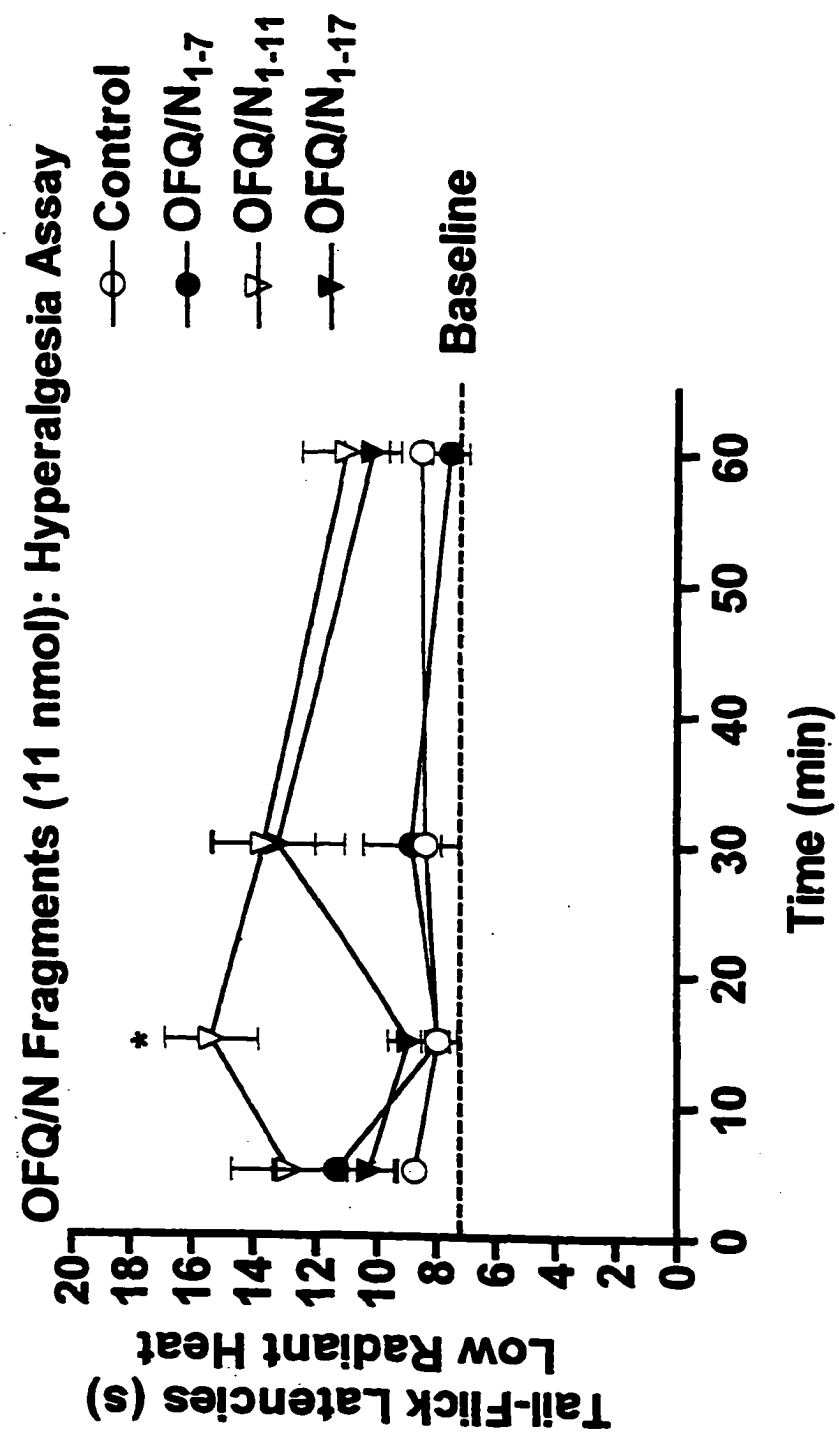
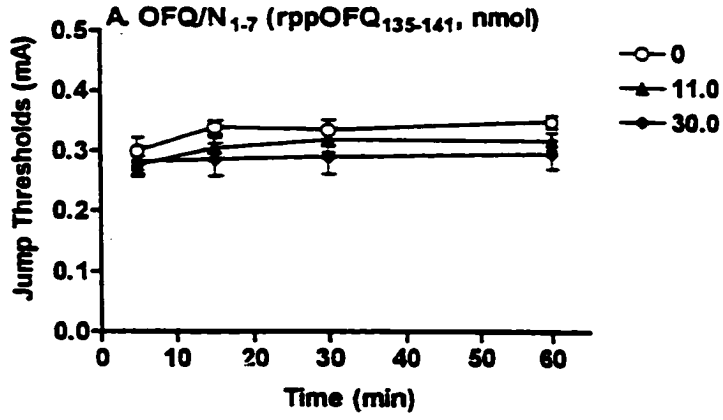
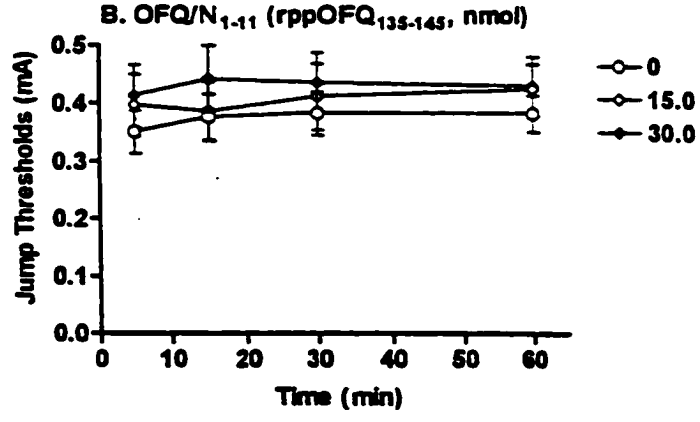
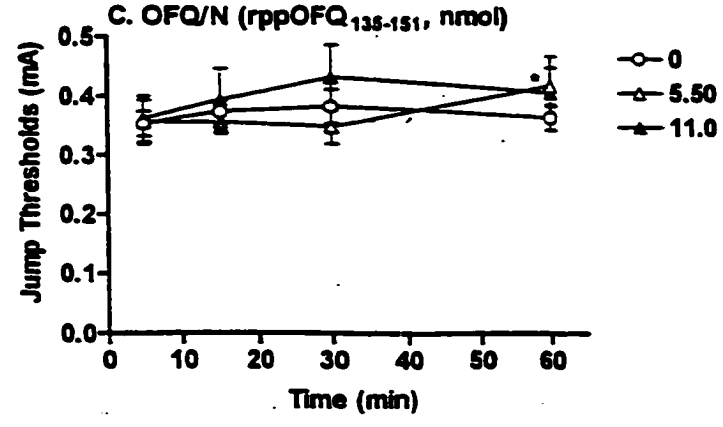


Figure 5. Alterations in jump thresholds (mA, ± S.E.M.) following administration of either rppOFQ/$N_{135-151}$, (upper panel) rppOFQ/$N_{135-145}$ (middle panel) or rppOFQ/$N_{135-141}$ (lower panel) into the amygdala relative to vehicle treatment.







NS) or for the interaction between conditions and times ($F(6,30)=0.64$, NS). Jump thresholds failed to be altered by either the 11- or 30-nmol doses of rppOFQ/$N_{135-141}$ in the amygdala (Fig. 5).

Discussion

The present study had the following three major findings. First, rppOFQ/$N_{135-151}$, and both of its truncated active fragments, rppOFQ/$N_{135-145}$ and rppOFQ/$N_{135-141}$, administered into the amygdala produced antinociception on the tail-flick test using high-intensity thermal stimuli set to produce short baseline latencies. The antinociceptive responses induced by the three OFQ/N fragments in the amygdala appeared to produce different dose-response profiles; the parent heptadecapeptide, rppOFQ/$N_{135-151}$, produces dose-dependent increases in tail-flick latencies between 0.15 and 0.74 nmol, which peaked at 1.5-3nmol, and then the magnitude of antinociception declined as the doses increased from 5.5 to 30 nmol. The antinociceptive magnitude (8-9.4 s) of rppOFQ/$N_{135-151}$ on the tail-flick test in the amygdala is considerably higher than those elicited by amygdala microinjections of either morphine, β-endorphin or U50 488H under similar testing circumstances (Pavlovic and Bodnar, 1998; Pavlovic et al., 1996). The dose-response curve for rppOFQ/$N_{135-151}$ is bell-shaped with some of the lower doses producing a greater magnitude of antinociception than the higher doses. This is possibly due to the higher doses producing a hyperalgesia, but the high-intensity tail-flick test cannot show this effect. Whereas rppOFQ/$N_{135-145}$ in the amygdala produced dose-dependent increases in high-intensity tail-flick latencies across a 1.5-3- nmol dose range, rppOFQ/$N_{135-141}$ in the amygdala significantly increased latencies following the lower (0.15-1.5 nmol), but not higher (11-30 nmol), doses. The peak magnitude (~5 s) of the

two truncated OFQ/N fragments, rppOFQ/$N_{135-145}$ and rppOFQ/$N_{135-141}$, in the amygdala were less than those observed for the parent peptide rppOFQ/$N_{135-151}$ (8-9.4 s) on the high-intensity tail-flick test; this pattern is similar to that observed following peripheral (Kolesnikov and Pasternak, 1999), intrathecal (King et al., 1997) and ventricular (Rossi et al., 1998) administration. Since rppOFQ/$N_{135-151}$ contains two pairs of basic amino acids, facilitating its further processing to either rppOFQ/$N_{135-145}$ or rppOFQ/$N_{135-141}$, it raises the possibility that the antinociception observed in the amygdala is due to activity of the fragments rather than the original peptide. However, the greater magnitude of rppOFQ/$N_{135-151}$-induced antinociception in the amygdala suggests that it retains its continued structural activity rather than undergoing this form of processing.

Second, neither rppOFQ/$N_{135-151}$, rppOFQ$N_{135-145}$ and rppOFQ/$N_{135-141}$ administered into the amygdala altered jump thresholds except for a transient (60 min) and small increase following the 5.5 nmol dose of rppOFQ/$N_{135-151}$, indicating test specific antinociceptive responses of OFQ/N fragments in the amygdala for thermal, but not electrical noxious stimuli. This profile of OFQ/N antinociception in the amygdala again indicates important differences from amygdala antinociception elicited by traditional opiate agonists. Thus, jump thresholds are markedly increased over a 120-min duration following comparable doses of either morphine, β-endorphin or U50 488H in the amygdala (Pavlovic and Bodnar, 1998; Pavlovic et al., 1996a; 1996b; Rodgers, 1977; 1978). These test-specific data suggest that the opioid receptors mediating traditional μ, κ and potentially δ agonists in the amygdala (Paden et al., 1987) probably have a different distribution than rppOFQ/$N_{135-151}$ and rppOFQ/$N_{135-145}$ binding sites identified in the amygdala using autoradiography (Letchworth et al., 2000). The jump test is a

measure of pain-inhibition, but there are also some motivational properties associated with this test, for example, the motivation to escape from the box. It is conceivable that the lack of effects seen on the jump test could be due to OFQ/N affecting motoric activity. However, Dr. Rossi (unpublished data) found that OFQ/N, at the doses used in this study, had no effect on locomotor activity.

Third, the amygdala does not appear to support any of the hyperalgesic effects of rppOFQ/$N_{135-151}$ or its fragments at least under the dose and testing situations employed in the present study. The original reports that OFQ/N produced hyperalgesia (Kavaliers and Perrot-Sinalts, 1996; Meunier et al., 1995; Reinscheid et al., 1995) were subsequently modified (Meunier, 2000) such that OFQ/N_{1-17} produced anti-opioid effects in both stress and opioid agonist antinociceptive assays (Grisel et al., 1996; Mogil et al., 1996a, 1996b, Tian et al., 1997). Given the selective deficits in opioid antinociceptive and opioid-mediated stress-induced analgesic responses following amygdala lesions (Fox and Sorenson, 1994; Helmstetter, 1993; Helmstetter et al., 1993; Manning and Mayer, 1995a; 1995b; Pavlovic et al., 1996a; Watkins et al., 1993), it would appear that this site would be a strong candidate for displaying such hyperalgesic responses. Since the traditional tail-flick paradigms using high-intensity thermal stimuli are not good indicators of hyperalgesia because of short baseline latencies, a less intense thermal stimulus was used to yield longer latencies. Yet, neither rppOFQ/$N_{135-151}$, rppOFQ/$N_{135-145}$ nor rppOFQ/$N_{135-141}$ decreased low-intensity tail-flick latencies following amygdala microinjections; indeed, the trend of the data for each OFQ fragment indicated further increases in these longer latencies. One might question whether this is a reliable measure of such effects. First, this procedure yields a reliable hyperalgesia in the mouse following

ventricular (Rossi et al., 1996), but not intrathecal (King et al., 1997) administration, suggesting a supraspinal mechanism of hyperalgesic action. Second, the profile of rppOFQ/$N_{135-151}$-induced hyperalgesia differs from that of rppOFQ/$N_{135-145}$-induced antinociception since antisense probes directed against coding exon 1, but not coding exons 2 and 3, of the ORL-1/KOR-3 clone block the former response while antisense probes directed against coding exons 2 and 3, but not coding exon 1, block the latter response (Rossi et al., 1997).

Two important caveats to the interpretation of these results is the volume of the microinjection which raises questions as to whether the site of action was actually in the amygdala. There are several lines of evidence which suggest, but cannot conclusively indicate the presence or absence of site-specificity. First, placements in both the central and basolateral nuclei of the amygdala produce similar patterns of dose-dependent and time-dependent agonist-induced effects, not only for the OFQ/N fragments in the present study, but for other opioid agonists reported previously (Helmstetter et al., 1993, 1995, 1998; Pavlovic and Bodnar 1998a, 1998b; Pavlovic et al., 1996; Rizvy et al., 1991; Rodgers, 1978; Tershner and Helmstetter, 2000). Therefore, either multiple nuclei in the amygdala mediate such effects, or agonist diffusion is occurring uniformly to one integral site within or outside of the amygdala. Second, there do not appear to be any profound differences between the agonists in their pharmacokinetic actions related to diffusion or their reactivity to lipids or water. Yet, as indicated, the OFQ/N fragments appear to display a different pattern of test-specific analgesic effects relative to morphine, DAMGO, β-endorphin and U50 488H. Although we did not perform a full mapping study, the ineffectiveness of a few misplaced cannula placements together with the

orderly dose-dependent and short-onset nature of OFQ/N fragment effects in verified amygdala placements suggest strongly that the amygdala is the site of action.

At least three possibilities exist for how OFQ/N is producing its effects. Firstly, it could be binding to an ORL-1/KOR-3 receptor. Secondly, it is conceivable that it is binding to one of the other traditional opioid receptors. While OFQ/N has low affinity for the traditional opioid receptors as compared to the ORL-1/KOR-3 receptor, it is still sufficient for some effects to take place (Gintzler et al., 1997). In either case, OFQ/N is activating descending pain inhibition and activating opioid neurons. We can't differentiate which possibility is occurring because no ORL-1/KOR-3 antagonist was used. There is an antagonist available, however, I could not get it. Finally, it is conceivable that OFQ/N is activating a disinhibiting neuron downstream (e.g. GABA).

Based on previous studies that showed that rppOFQ/$N_{135-151}$ produces pro-nociceptive, hyperalgesic, anti-opioid, anti-stress or antinociceptive effects, I thought that OFQ/N injections into the amygdala would produce a few of these effects when tested on the high-intensity tail-flick, low-intensity tail-flick and jump tests. However, I did not find this to be the case. On all nociceptive assays used OFQ/N seemed to produce either antinociceptive effects or no effect at all. One possibility may be that a lot of doses were tested on the high-intensity tail-flick test, which tested for the antinociceptive properties of the OFQ/N fragments, whereas fewer doses of OFQ/N were used to test for hyperalgesic effects (the low-intensity tail flick test) and antinociceptive effects on other nociceptive assays (jump test). It is conceivable that some other doses would have produced other effects, possibly hyperalgesia on the low-intensity tail-flick test, or antinociception on the jump test. There are several reasons why this was not done in this

experiment. Firstly, OFQ/N was expensive and hard to get. The peptide had to be prepared daily just before testing, and had to be discarded at the end of the testing session due to the instability of the peptide. So, when no effects were seen on the initial doses used for the low-intensity tail-flick and jump test, these two tests were aborted. Also, the time course used during this experiment made it prohibitive to use more than one nociceptive assay since each animal was tested five minutes post-agonist injection. Another possibility may be due to method differences among different studies relative to the state of the animal. In all my studies the animals were habituated (e.g., handling and repeated testing) to all nociceptive assays used so the animals were not stressed. Other studies may not have habituated their animals thus inducing some sort of stressful state, and then testing the animal on a nociceptive assay.

The next study provides further support for the present set of data indicating that OFQ/N peptide effects occurred within the amygdala. The precursor responsible for producing rppOFQ/$N_{135-151}$ (Houtani et al., 1996; Meunier et al., 1995; Pan et al., 1996) yields rppOFQ/N2 (rppOFQ/$N_{154-170}$) which also produces antinociception (Rossi et al., 1998). In analyzing the peptide fragments responsible for rppOFQ/N2-induced antinociception, we (Shane et al., 1999) found that rppOFQ/$N_{154-181}$ produces antinociceptive *and* hyperalgesic responses using high-intensity and low-intensity tail-flick assays following microinjections into the locus coeruleus and RVM, but produces antinociceptive *without* hyperalgesic responses using these procedures following microinjections into the vlPAG. The next study examines the effects of rppOFQ/$N_{154-181}$ upon antinociceptive *and* hyperalgesic responses using high-intensity and low-intensity tail-flick assays as well as the jump test.

CHAPTER 4: SPECIFIC AIM TWO

rppOFQ/$N_{154-181}$ Antinociception and Hyperalgesia Elicited from the Amygdala:

Introduction

This study evaluated the antinociceptive and hyperalgesic effects of a related peptide, rppOFQ/$N_{154-181}$ in the amygdala of rats on both radiant heat and shock tests according to a similar strategy to that employed in the first study. This peptide is at the end of the pre-pro Orphanin precursor gene. Initial studies with this and other related peptides found that it elicited similar antinociceptive effects to rppOFQ/$N_{135-151}$ when given i.c.v. in mice (Rossi et al., 1998). The present study examining the rppOFQ/$N_{154-181}$ in the amygdala was part of a larger study evaluating the effects of this peptide in other supraspinal sites as well including the vlPAG, RVM and LC.

Methods

Three different protocols were employed and are summarized in Table 2. First, a dose-response (0.005-30 μg) curve for rppOFQ/$N_{154-181}$-induced antinociception in the amygdala was assessed on the high intensity tail-flick test. Then a dose (20 μg) of rppOFQ/$N_{154-181}$ was assessed for hyperalgesia on the low-intensity tail-flick test as well as the jump test. Finally, the ability of systemic administration of the opioid antagonist, naloxone (5 mg/kg) to attenuate OFQ/$N_{154-181}$ in the amygdala was evaluated. In all three protocols, high-intensity and low-intensity tail-flick latencies and jump thresholds were determined 5, 15, 30 and 60 minutes following vehicle treatment and agonist microinjections. In protocol C, naloxone (5mg/kg) was given systemically 15 minutes before rppOFQ/$N_{154-181}$

Table 2. Summary of experimental groups in Experiment 2.

A. rppOFQ/$N_{154-181}$-induced antinociception on high-intensity tail flick test:

	μg	nmol	n
	Vehicle		34
	0.005	0.0015	6
	0.05	0.015	6
	0.5	0.15	6
	2.5	0.75	6
	5	1.5	6
	10	3	6
	20	6	7
	30	9	6

B. Low-intensity tail flick test (hyperalgesia):

	Vehicle		6
rppOFQ/$N_{154-181}$	20	6	6

C. Jump test:

	Vehicle		6
rppOFQ/$N_{154-181}$	20	6	6

D. Pretreatment with Naloxone systemically with a fixed dose of rppOFQ/$N_{154-181}$ (20μg/μl) in amygdala.

	mg/kg	n
	Vehicle	12
	0.5	6
	5.0	6

injection in the amygdala. There was a one week interval between particular conditions; this interval minimized agonist-induced tolerance effects. No animal in any protocol received more that five microinjection conditions in a given site.

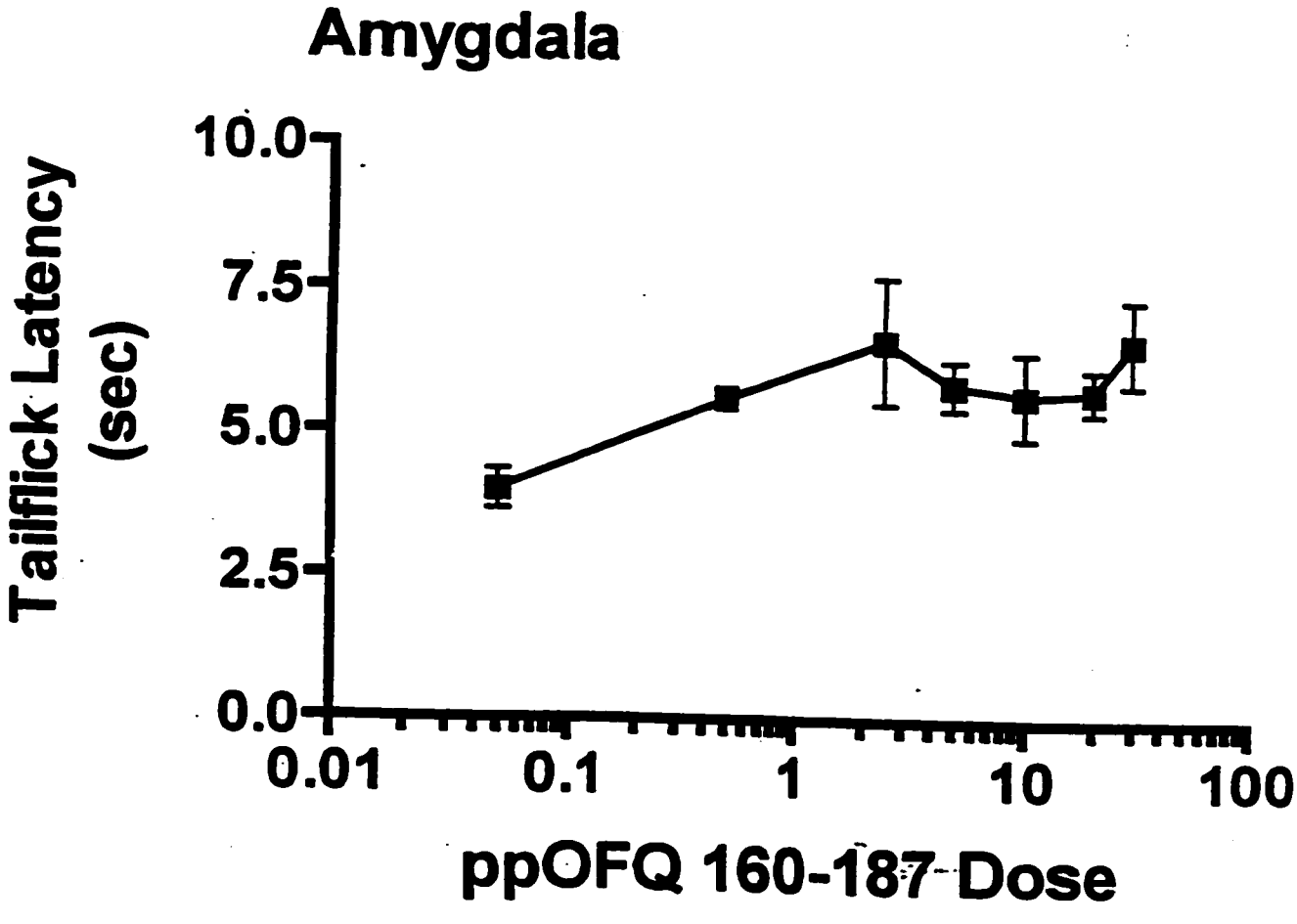
Results

Histological verification. All the rats in the treatment protocols had cannula placements in the central and baso-lateral nuclei of the amygdala. The placements of the cannulae across paradigms were quite similar, and also similar to those in Experiment 1. No significant differences were observed in the magnitude of OFQ/N-induced antinociception between nuclei, and thus data for each agonist treatment for the two amygdala placements were pooled.

Amygdala rppOFQ/$N_{154-181}$ and the high-intensity tail-flick assay. Significant differences were observed among experimental conditions ($F(7,56)=116.89$, $P<0.0001$), across test times ($F(3,168)=17.04$, $P<0.0001$) and for the interaction between conditions and times ($F(21,168)=2.78$, $P<0.0001$). A wide range (0.0015-9 nmol) of rppOFQ/$N_{154-181}$ doses in the amygdala significantly increased high-intensity tail-flick latencies with effects observed up to 5 minutes following the 0.15 nmol dose, up to 15 min following the 9 nmol dose and across the 60-min time course following the 0.75-6 nmol dose ranges (Fig. 6). The magnitude of rppOFQ/$N_{154-181}$-induced antinociceptive effects in the amygdala displayed an inverted U-shaped function such that latencies increased from 0.15 nmol, peaked at 0.75-6 nmol, and then declined in antinociceptive efficacy from 9 nmol.

Amygdala rppOFQ/$N_{154-181}$ and the low-intensity tail-flick assay. Significant differences were observed among conditions ($F(1, 10)=7.12$, $P<0.0235$) and across

Figure 6. **Alterations in high-intensity tail-flick latencies following administration of rppOFQ/$N_{154-181}$ into the amygdala across a wide dose range (0.015-30 nmol) relative to vehicle treatment. Peak effects at 5 min are shown.**



times ($F(2, 2)=2.64$, $P<0.256$), but not for the interaction between conditions and times ($F(2,20)=0.40$, NS). Amygdala microinjections of rppOFQ/$N_{154-181}$ peptide increased low-intensity latencies rather than produce decreases in these latencies as expected for the presence of a hyperalgesic state (Fig. 7).

Amygdala rppOFQ/$N_{154-181}$ and jump thresholds. Significant differences in the rppOFQ/$N_{154-181}$ paradigm were observed across test times ($F(3,36)=3.66$, $P<0.021$) but not among conditions ($F(1, 12)=0.04$, NS), and the interaction between conditions and time ($F(3,36)=0.77$, NS). Jump thresholds failed to be altered by rppOFQ/$N_{154-181}$ in the amygdala following the 6-mol dose (Fig. 8).

rppOFQ/$N_{154-181}$ Antinociception and Opioid Receptor Antagonism in the Amygdala. Significant differences were observed among experimental conditions ($F(3,33)=167.96$, $p<0.0001$), across test times ($F(2, 22)=17.65$, $p<0.0001$), and for the interaction between conditions and times ($F(6, 66)=2.32$, $p<0.043$). rppOFQ/$N_{154-181}$ administered into the amygdala significantly increased latencies across the 60-min time course relative to vehicle treatment (Fig. 9). Naloxone significantly reduced the magnitude of rppOFQ/$N_{154-181}$ antinociception elicited from the amygdala following agonist treatment for the low and high antagonist doses (Fig. 9).

Discussion

The results of the present study indicated that rppOFQ/$N_{154-181}$ produced dose-dependent analgesic responses on the high-intensity tail-flick test following microinjection into the PAG, amygdala, LC and RVM, indicating multiple sites of supraspinal action. Although nalaxone was capable of eliminating rppOFQ/$N_{154-181}$ - induced analgesia in the PAG and amygdala, it generally failed to alter this

Figure 7. **Alterations in low-intensity tail-flick latencies (s, ±S.E.M.) following administration of rppOFQ/$N_{154-181}$ into the amygdala relative to vehicle treatment.**

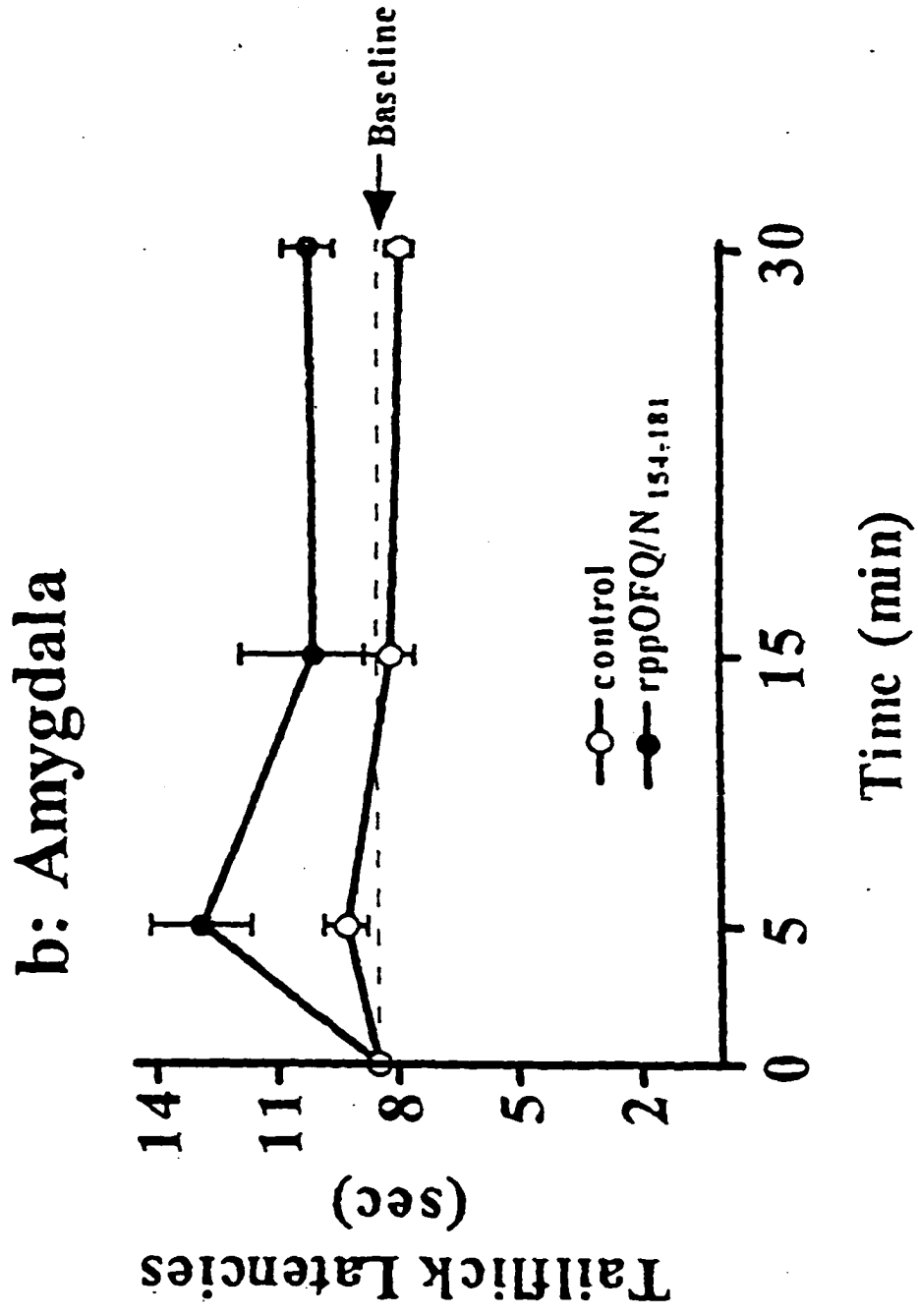


Figure 8. **Alterations in jump thresholds (mA, ±S.E.M.) following administration of rppOFQ/$N_{154-181}$ (20 μg), into the amygdala relative to vehicle treatment.**

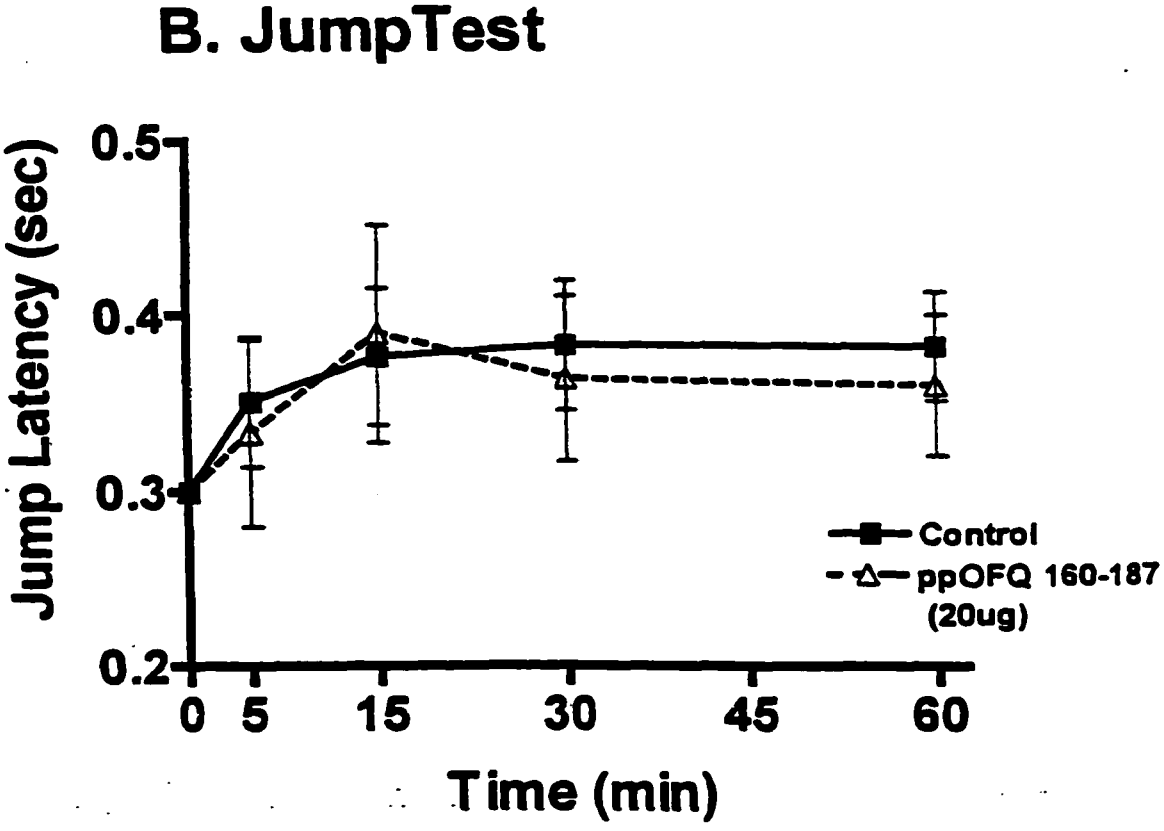
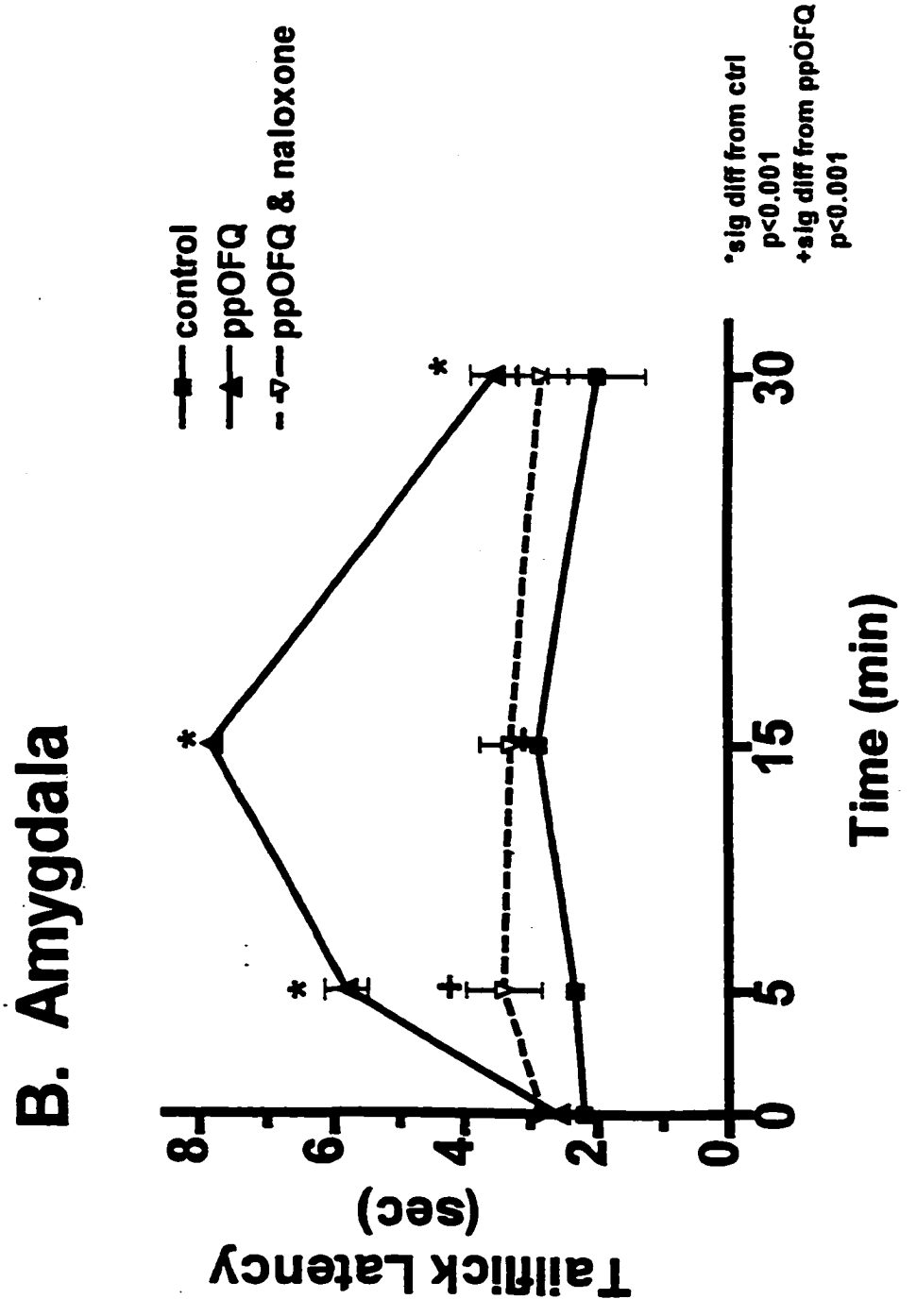


Figure 9. Alterations in high-intensity tail-flick latencies following administration of rppOFQ/$N_{154-181}$ (20 μg), or pretreament with Naloxone (5mg/kg, s.c.) prior to rppOFQ/$N_{154-181}$ into the amygdala of rats. The crosses in this and subsequent figures denote significant decreases in latencies relative to corresponding OFQ/N values.



analgesic response in the LC and RVM, suggesting site-specific pharmacological mechanisms of action. Although rppOFQ/$N_{154-181}$ was capable of producing a hyperalgesic response in the LC and RVM when tested at the low-intensity tail-flick test, it failed to produce hyperalgesia under these conditions in the PAG and amygdala, suggesting different site-specific mechanisms of action. Therefore, these data indicate site-specificity mediating the mechanism of antinociceptive actions and hyperalgesia. The presence of this double- dissociation serves as an important control in analyzing the specificity of the responses. These pairs of sites behave quite similarly in eliciting traditional opioid antinociception with greatest sensitivity to mu-selective ligands (Bodnar et al., 1988, Smith et al., 1988, Pavlovic et al., 1996, Fang et al., 1986, Helmstetter et al., 1995) as well as exhibit antinociceptive opioid synergy using morphine and β-endorphin (Rossi et al., 1993, 1994; Pavlovic and Bodnar, 1998).

Interestingly, in the four rat brain sites examined, rppOFQ/$N_{154-181}$ produced potent antinociception. However, the antinociceptive response in two of the cannulated sites (amygdala and PAG) was naloxone-reversible. Moreover, those sites that did not produce an opiate-reversible antinociception (the LC and the RVM), showed significant hyperalgesia. Is it possible for the same brain region to produce two different effects, both antinociception and pronociception? Previous research shows that pronociceptive and antinociceptive effects of Orphanin FQ/Nociceptin (OFQ/N) are dissociable. The first line of evidence comes from earlier studies in our laboratory showing that antisense which targets the coding region of exon 1 of the ORL1 receptor clone decreases OFQ/N hyperalgesia when given icv, however, antisense targeting exons 2 and 3 of the same clone are ineffective icv. In contrast, ORL1 antisense to exons 2 and 3 decrease OFQ/N

icv antinociception, while ORL1 exon 1 is ineffective in the RVM and LC (Rossi et al., 1997). In addition, other laboratories have confirmed the possibility of dissociation in a single brain region. In 1991, the Fields laboratory, showed an interesting line of evidence that supports this dissociation in the RVM which is highly compelling (Fields et al., 1991). Fields and his co-workers demonstrated ON-OFF cells in the RVM which were both pain-facilitory and pain-inhibitory systems. This neurophysiological evidence for the role of pro-nociceptive and antinociceptive dissociation is remarkably seen in further reports from Dr. Heinricher's laboratory (Heinricher et al., 1999; Heinricher and Roychowdhury, 1997). Our data now implicate RVM rppOFQ/$N_{154-181}$ in this dissociation. Interestingly, this pain-facilitory and pain-inhibitory dichotomy of cell neurophysiological responsivity is not apparent in the PAG. Our data now confirms this. Hyperalgesia was not elicited from the PAG, merely antinociception in the PAG and amygdala. Therefore, the present study demonstrates that it is possible to produce both pronociceptive and antinociceptive effects following rppOFQ/$N_{154-181}$ microinjection. Moreover, this possibility of opiate or non-opiate action is clearly based upon the supraspinal site of action.

The opioid receptor-like (ORL1) receptor is structurally related to opioid receptors and the ORL1 receptor agonist, OFQ/N, induces antinociception at the spinal level, but appears to recruit different circuitry than that used by traditional μ opioids (Monteillet-Agius, 1999). The functionally distinct actions of antinociception and hyperalgesia in the ORL1 receptors may be activated by rppOFQ/$N_{154-181}$ by functionally distinct intracellular signaling systems, as well as being explained by their differential cellular localization. Interestingly, the ability of the full-length peptide, rppOFQ/$N_{154-181}$

to induce hyperalgesia is site-specific with caudal sites (LC and RVM) mediating this response. This suggests that different functional and physiological mechanisms other than the amygdala and PAG may mediate the pro-nociceptive effects of the full-length peptide in the rostral part of the brain.

The ability of naloxone to reverse the antinociceptive actions of the full-length peptide (Rossi et al., 2002; Gintzler et al., 1997) raises interesting questions. We have shown that both the full-length peptide (rppOFQ/$N_{154-181}$) and the N-terminal fragment (rppOFQ/$N_{154-170}$) possess bioactivity, with the parent peptide showing more potency in most physiological tests. In contrast, the small C-terminal peptide shows no bioactivity. Nevertheless, the minimum peptide sequence for complete bioactivity is not known at present, but nonetheless active in the brain.

The biochemical isolation of rppOFQ/$N_{154-181}$, combined with the demonstration of its complex physiological activities, provides evidence that biologically active peptides are processed from the precursor peptide, ppOFQ/N. Studies by Danielson et al., (2001) describe the cloning of OFQ and provide further evidence that ppOFQ/N evolved from a prodynorphin-like molecule and that the proOFQ/N molecule has no homologous C-terminal peptide, indicating that the mouse, rat and human evolved separate, distinct peptides that are identical at the amino acid level. These studies from the Danielson and Dores' laboratory, in addition to ours, provide insight into the underlying mechanisms by which novel, and often divergent, physiological functions arise in opioid and non-opioid systems (Danielson, et al., 2001). Therefore, it is possible and probable, that the OFQ/N system may have evolved as a separate and distinct system for pain perception.

Thus, this related OFQ/N peptide seems to share very similar characteristics relative to rppOFQ/$N_{135-151}$ and its truncated fragments as described in Chapter 3. This suggests that perhaps all of the biologically-active fragments of ppOFQ/N may act similarly with respect to modulating antinociceptive and pro-nociceptive actions. To explore the relationships of these interesting peptides with traditional opioid systems, the next experiment examined whether pretreatment with general or selective opioid antagonists would affect the antinociceptive response to the originally-described hepadecapeptide, rppOFQ/$N_{135-151}$.

CHAPTER 5: SPECIFIC AIM THREE

rppOFQ/$N_{135-151}$-Induced Antinociception Elicited from the Amygdala: Role of Amygdala Opioid Antagonists

Introduction

The present study determined whether rppOFQ/$N_{135-151}$ antinociception in the amygdala can be blocked by pretreatment in the same amygdala site with equimolar doses of either general (naltrexone), μ (β-FNA), κ (NBNI) or δ (NTI) opioid receptor subtypes in rats.

Methods

Table 3 summarizes the number and sample sizes of the different experimental groups. OFQ/N_{1-17} (5.5 μg, 3 nmol, 0.1 μl) was injected into the amygdala and high-intensity tail-flick latencies were assessed. This dose produced the most pronounced analgesic effects over a 0.027-55 μg dose-response curve over a similar time course (Shane et al., 2001). Subgroups of rats, matched both for vehicle tail-flick latencies and for magnitude of rppOFQ/$N_{135-151}$-induced antinociception, received either NTX at doses of either 0.1 or 1.0 μg, β-FNA at doses of either 0.133 or 1.33 μg, NBNI at doses of either 0.2 or 2.0 μg, and NTX at doses of either 0.135 or 1.35 μg prior to rppOFQ/$N_{135-151}$ in the amygdala. Tail-flick latencies were determined 5, 15, 30 and 60 minutes following rppOFQ/$N_{135-151}$ microinjections. Each of the high (2.7 nmol) and each of the low (0.27 nmol) antagonist doses were equimolar with respect to one another. The dose range of each selective antagonist has previously been shown to block intracerebral antinociception elicited by a receptor-selective agonist: DAMGO-induced antinociception by β-FNA,

Table 3. Summary of experimental groups in Experiment 3.

rppOFQ/$N_{135-151}$-induced antinociception (5.5 μg, 3nmol) and opioid antagonists in the amygdala:

Antagonist	Dose (μg/nmol)	n
Vehicle		25
NTX	0.1/0.27	6
NTX	1.0/2.7	6
β-FNA	0.133/0.27	6
β-FNA	1.33/2.7	6
NBNI	0.2/0.27	6
NBNI	2.0/2.7	6
NTI	0.135/0.27	6
NTI	1.35/2.7	6

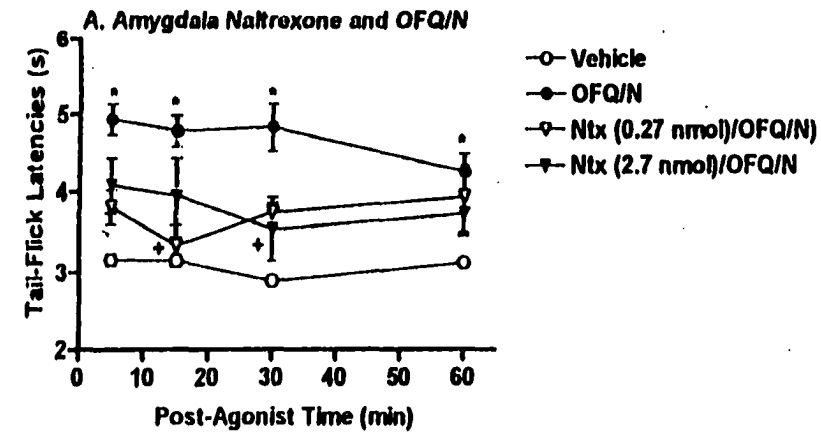
deltorphin-induced antinociception by NTI, and U50 488H-induced antinociception by NBNI (Rossi et al., 1994; Pavlovic and Bodnar, 1998).

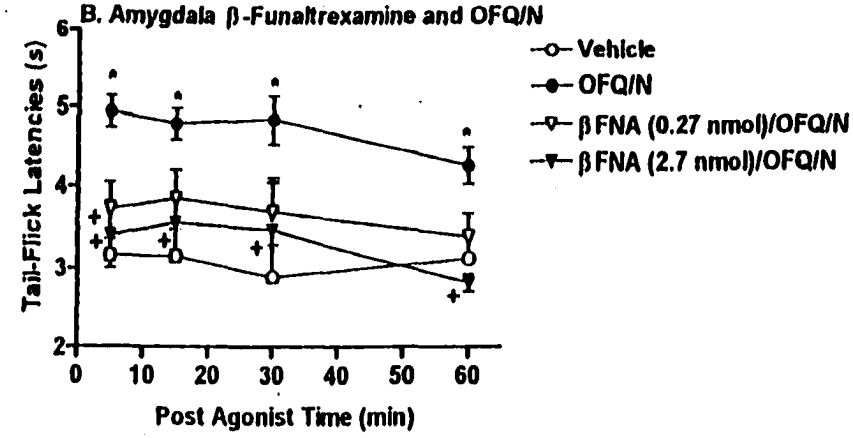
Results

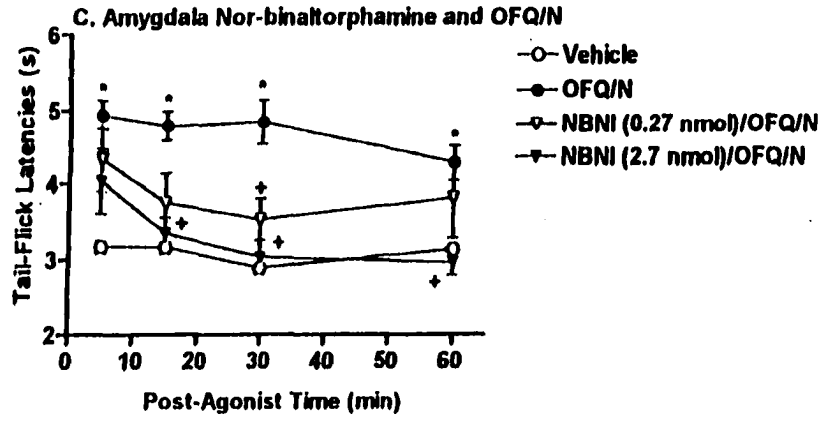
Histological Verification. The 25 rats in the treatment protocols had cannula placements in the medial, central and baso-lateral nuclei of the amygdala similar to those found in previous studies (Pavlovic et al., 1996; 1998a; 1998b; Rossi et al, 2002; Shane et al., 2001) and the previous two chapters. Since no significant differences were observed in the magnitude of rppOFQ/$N_{135-151}$ antinociception between nuclei, these data were pooled.

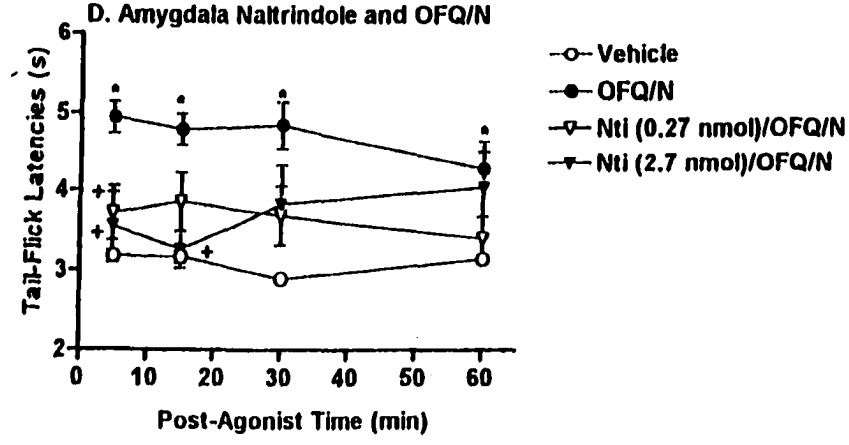
rppOFQ/$N_{135-151}$ Antinociception and Opioid Receptor Antagonism in the Amygdala. Significant differences were observed among experimental conditions ($F(9,88)=3.41$, $p<0,0012$), across test times ($F(3, 264)=2.77$, $p<0.042$), but not for the interaction between conditions and times ($F(27, 264)=1.06$, n.s.). rppOFQ/$N_{135-151}$ administered into the amygdala significantly increased latencies across the 60-min time course relative to vehicle treatment (Fig. 10), an effect that was not observed for antagonist-rppOFQ/$N_{135-151}$ pairings. NTX significantly reduced the magnitude of rppOFQ/$N_{135-151}$ antinociception elicited from the amygdala following agonist treatment for the low (15 min) and high (30 min) antagonist doses (Fig. 10, Panel A). The μ-selective antagonist, β-FNA dose-dependently and significantly reduced rppOFQ/$N_{135-151}$ antinociception elicited from the amygdala with the low dose producing transient (5 min) effects and the high dose effective across the entire time course (Fig. 10, Panel B). The κ- selective antagonist, NBNI dose-dependently and significantly reduced rppOFQ/$N_{135-151}$ antinociception elicited from the amygdala with the low dose, producing transient

Figure 10. Alterations (Mean, +S.E.M.) in tail-flick latencies in rats receiving vehicle, rppOFQ/$N_{135-151}$ (5.5 μg), or pretreatment with either naltrexone (NTX, Panle A), β-funaltrexamine (βFNA, Panel B), nor-binaltorphamine (NBNI, Panel C) or naltrindole (NTI, Panel D) prior to rppOFQ/$N_{135-151}$ into the amygdala of rats.









(30 min) effects and the high dose effective 15-60 min after agonist treatment (Fig. 10, Panel C). The δ-selective antagonist, NTI significantly reduced rppOFQ/$N_{135-151}$ antinociception elicited from the amygdala 5 min following the low dose and 5-15 min following the high dose (Figure 10, Panel D).

Discussion

The present study indicated that antinociception elicited by rppOFQ/$N_{135-151}$ administered in the amygdala is dependent upon the intact functioning of μ, κ and δ opioid receptors in the amygdala since pretreatment with either general or selective opioid antagonists in the amygdala significantly reduced this agonist's antinociceptive response. Three possibilities exist to explain these effects. First, it is conceivable that rppOFQ/$N_{135-151}$ is binding directly with μ, κ or δ receptors in the amygdala (Mansour et al., 1987; 1994a; 1994b; 1994c; 1995a; 1995b; 1996) to elicit antinociceptive effects rather than to the ORL-1/KOR-3 receptor in the amygdala (Anton et al., 1996; Letchworth et al., 2000; Neal et al., 1999a). This possibility is unlikely because definitive initial characterization of rppOFQ/$N_{135-151}$ showed that it binds poorly to traditional μ, κ and δ opioid receptors as compared to its high affinity for ORL-1/KOR-3 receptors (Butour et al., 1997; Meunier et al., 1995; Reinscheid et al., 1995). Second, it is conceivable that the four antagonists (NTX, β-FNA, NBNI, NTI) are successfully competing with rppOFQ/$N_{135-151}$ in the amygdala to act at the ORL-1/KOR-3 receptor rather than acting at their respective traditional opioid receptor subtypes. This possibility is unlikely given the high affinity of rppOFQ/$N_{135-151}$ for the ORL-1/KOR-3 receptor (Butour et al., 1997; Meunier et al., 1995; Reinscheid et al., 1995) relative to the poor and even negligible affinity for the ORL-1/KOR-3 receptor of traditional μ (e.g., morphine,

fentanyl) κ (dynorphin A(1-8), tifluadom) or δ (e.g., D-Pen2, D-Pen5-enkephalin) agonists which are readily and respectively reversed by β-FNA, NBNI and NTI (Bunzow et al., 1994; Mollereau et al., 1994, Pan et al., 1994; Pan et al., 1995; Wick et al., 1994). Whereas these first two possibilities invoke antagonist-agonist interactions at common receptor sites within the amygdala, the third possibility posits physiological interaction in the amygdala between neurons with traditional opioid receptors (Mansour et al., 1987; 1994a; 1994b; 1994c; 1995a; 1995b; 1996) and neurons with the ORL-1/KOR-3 receptor (Anton et al., 1996; Letchworth et al., 2000; Neal et al., 1999a) as well as the localization of rppOFQ/$N_{135-151}$ to the amygdala (Neal et al, 1999b). These studies reveal that the medial amygdaloid nucleus is a common site at which dense distributions of traditional μ, κ and δ opioid receptors. However, the autoradiographic and mRNA techniques used to visualize these receptors in the amygdala do not specify their synaptic distribution so it is not clear whether such interactions occur at the same neuron or at trans-synaptically linked neurons. Electron microscopic evidence of opioid receptor distributions within the amygdala is limited to findings that μ-opioid receptors are on efferents from the central nucleus of the amygdala to the nucleus of the solitary tract (Pickel and Colago, 1999). Nevertheless, the present evidence strongly suggests that rppOFQ/$N_{135-151}$ produces antinociception elicited from the amygdala by activating traditional opioid receptor synapses within the same structure.

The next study examined whether traditional opioid receptors mediated OFQ/N-induced antinociception elicited from the amygdala through synapses in another highly-related structure, the vlPAG, and whether this regional interaction between sites occurred in ascending as well as descending directions.

CHAPTER 6: SPECIFIC AIM FOUR

rppOFQ/$N_{135-151}$-Induced Antinociception Elicited from the Amygdala or the vlPAG: Role of Opioid Antagonists in the Other Site

Introduction

The present study evaluated whether rppOFQ/$N_{135-151}$ antinociception elicited from the amygdala can be blocked by pretreatment with equimolar doses of general (naltrexone), μ (β-FNA), κ (NBNI) and δ (NTI) opioid receptor subtypes in the vlPAG in rats to indicate whether this peptide utilizes a similar anatomical and functional pathway to that of the traditional opioid agonists. Site-specificity of any positive antagonist effects within the vlPAG were assessed by determining antagonist effectiveness in misplaced mesencephalic placements. Finally, to examine reciprocal ascending influences, the ability of selective opioid antagonists administered into the amygdala were assessed for effects upon rppOFQ/$N_{135-151}$-induced antinociception elicited from the vlPAG.

Methods

Four different protocols were employed and are summarized in Table 4: a) opioid antagonists in the vlPAG and rppOFQ/$N_{135-151}$ in the amygdala, b) opioid antagonists in misplaced mesencephalic placements and rppOFQ/$N_{135-151}$ in the amygdala, c) opioid antagonists in the amygdala and rppOFQ/$N_{135-151}$ in the vlPAG, and d) opioid antagonists in the amygdala or the vlPAG. In Protocols A-C, tail-flick latencies were assessed at 5, 15, 30 and 60 minutes following rppOFQ/$N_{135-151}$ microinjections. In Protocol D, tail-flick latencies were assessed at 25, 35, 50 and 80 minutes following antagonist microinjection to adjust for when naltrexone was administered prior to the agonist.

Table 4. Summary of experimental groups in Experiment 4:

A. Opioid antagonists in the vlPAG and rppOFQ/$N_{135-151}$ (5.5 μg, 3nmol) in amygdala:

Antagonists	Dose (μg)	n
Vehicle		21
NTX	0.1	6
NTX	1.0	7
β-FNA	0.133	6
β-FNA	1.33	7
NBNI	0.2	6
NBNI	2.0	7
NTI	0.135	6
NTI	1.35	6

B. Opioid antagonists in misplaced mesencephalic site and rppOFQ/$N_{135-151}$ (5.5 μg, 3nmol) in amygdala:

Antagonists	Dose (μg)	n
Vehicle		7
NTX	1.0	5
β-FNA	1.33	5
NBNI	2.0	5
NTI	1.35	5

C. Opioid antagonists in bilateral amygdala and rppOFQ/$N_{135-151}$ (10 μg, 5.5 nmol) in the vlPAG:

Antagonists	Dose (μg)	n
Vehicle		9
NTX	0.5	5
β-FNA	0.665	7
NBNI	1.0	6
NTI	0.675	7

D. NTX in amygdala or vlPAG:

Amygdala	n=4
VlPAG	n=4

Double-injection Procedures: Protocols A and B utilized this procedure. Each rat received a maximum of four double microinjections at weekly intervals. The first injection was pretreatment of the vlPAG or misplaced mesencephalic cannula placement with general (NTX) or selective (β-FNA, NBNI, and NTI) opioid antagonists at a previously established dose (1.0 μg, 1.33 μg, 2.0 μg and 1.35 μg, respectively). The second injection was rppOFQ/$N_{135-151}$ (5.5 μg) into the amygdala.

Triple-Injection Procedures: Protocol C utilized this procedure. Each rat received a maximum of four triple injections at weekly intervals. The first two injections were pretreatment of bilateral amygdala with general (NTX) or selective (β-FNA, NBNI, and NTI) opioid antagonists at a previously established dose mentioned above (half of each dose was given into each amygdala placement). The third injection was rppOFQ/$N_{135-151}$ (10 μg) into the vlPAG. This rppOFQ/$N_{135-151}$ dose produced the most pronounced antinociceptive effects in the vlPAG in previous studies (data not published).

Results

Histological Verification

All rats in the treatment protocols had cannula placements in the medial, central and baso-lateral nuclei of the amygdala similar to those found in previous studies (Pavlovic and Bodnar, 1998a, 1998b; Pavlovic et al., 1996; Rossi et al., 2002; Shane et al., 2001). Since no significant differences were observed in the magnitude of rppOFQ/$N_{135-151}$ antinocieption between nuclei, these data were pooled.

vlPAG Naltrexone and rppOFQ/$N_{135-151}$ Antinociception elicited from the Amygdala: Significant differences were observed among experimental conditions ($F(4, 60) = 44.21$, $p<0.0001$), across test times ($F(3, 45) = 5.58$, $p<0.002$) and for their

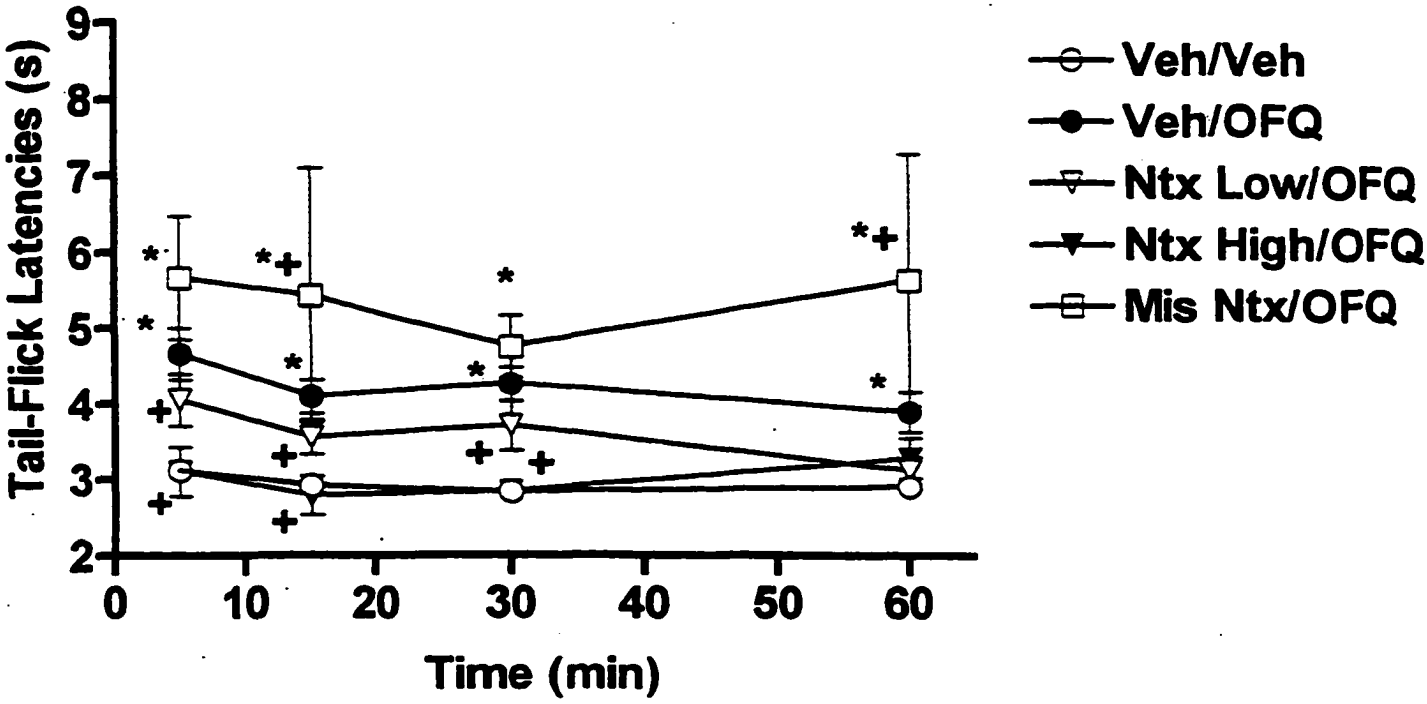
interaction ($F(12, 180) = 2.47$, $p<0.005$). rppOFQ/$N_{135-151}$ administered into the amygdala significantly increased latencies across the 60-min time course relative to vehicle treatment (Fig. 11, upper panel). The magnitude of rppOFQ/$N_{135-151}$ antinociception elicited from the amygdala was significantly reduced after 5, 15 and 30 min following vlPAG pretreatment with either the lower or higher doses of naltrexone. In contrast, naltrexone pretreatment in misplaced mesencephalic sites failed to reduce amygdala rppOFQ/$N_{135-151}$ antinociception, and indeed enhanced this response after 15 and 60 min.

vlPAG β-FNA and rppOFQ/$N_{135-151}$ Antinociception elicited from the Amygdala: Significant differences were observed among experimental conditions ($F(4, 60) = 72.07$, $p<0.0001$), across test times ($F(3, 45) = 3.50$, $p<0.023$) and for their interaction ($F(12, 180) = 4.84$, $p<0.0001$). rppOFQ/$N_{135-151}$ administered into the amygdala significantly increased latencies after 5, 15 and 30 min relative to vehicle treatment (Fig. 11, lower panel). In contrast to naltrexone's effects, the magnitude of rppOFQ/$N_{135-151}$ antinociception elicited from the amygdala was significantly reduced only after 5 min following vlPAG pretreatment with only the higher dose of β-FNA. β-FNA pretreatment in the vlPAG at both doses prevented the expression of significant rppOFQ/$N_{135-151}$ antinociception elicited from the amygdala. In contrast, rats receiving β-FNA pretreatment in misplaced mesencephalic sites displayed significant amygdala rppOFQ/$N_{135-151}$ antinocieption with this response enhanced after 60 min.

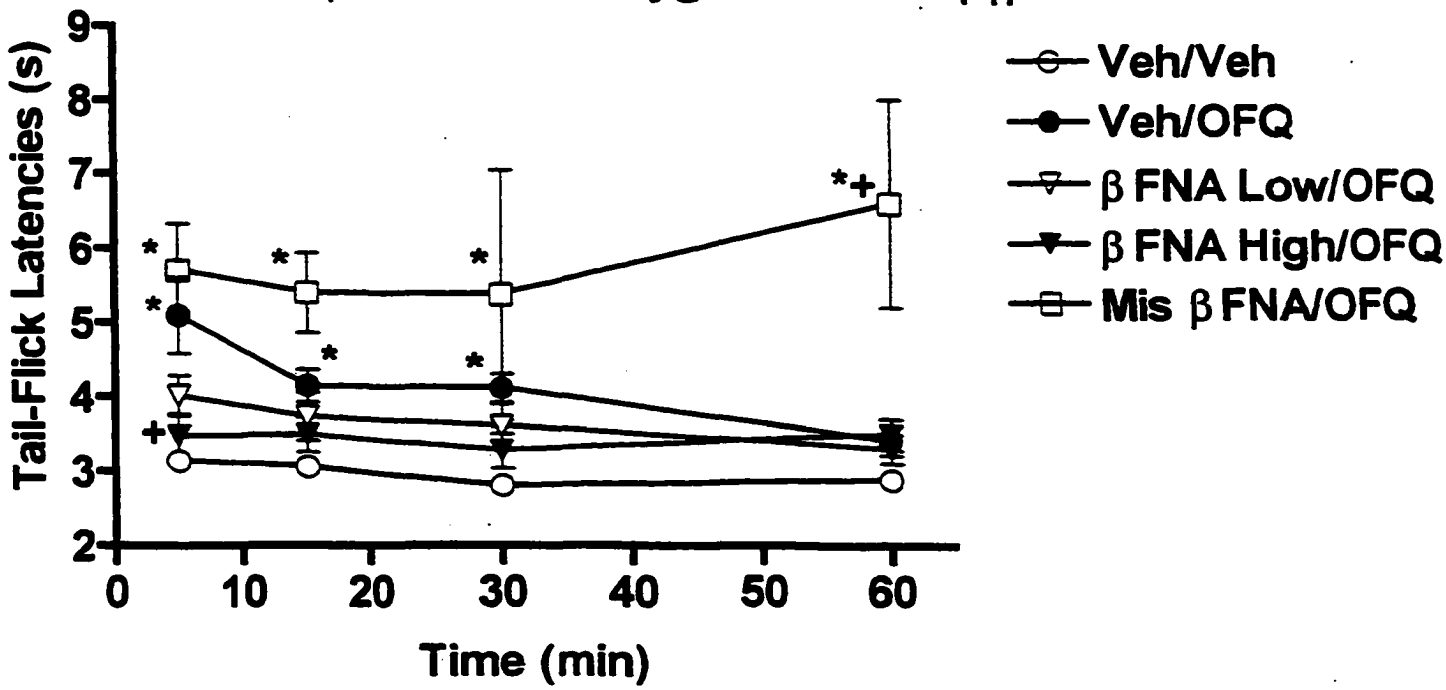
vlPAG NBNI and rppOFQ/$N_{135-151}$ Antinocciception elicited from the Amygdala: Significant differences were observed among experimental conditions ($F(4, 56) = 71.30$, $p<0.0001$), for the interaction between conditions and times ($F(12, 168) = 4.51$, $p<0.0001$), but not across test times ($F(3, 42) = 1.94$, n.s.).rppOFQ/$N_{135-151}$

Figure 11. Alterations (Mean, ±SEM) in tail-flick latencies in rats receiving vehicle, rppOFQ/$N_{135-151}$ (5.5 μg) in the amygdala, or pretreatment with either naltrexone (NTX, Panel A) or β-funaltrexamine (β-FNA, Panel B) in the vlPAG prior to rppOFQ/$N_{135-151}$ into the amygdala of rats.

A. vlPAG Naltrexone and Amygdala OFQ/N$_{1-17}$



B. vlPAG β-FNA and Amygdala OFQ/N$_{1-17}$



administered into the amygdala significantly increased latencies after 5, 15 and 30 min relative to vehicle treatment (Fig. 12, upper panel). In contrast to naltrexone's effects, the magnitude of rppOFQ/$N_{135-151}$ antinociception elicited from the amygdala was significantly reduced only after 5 min following vlPAG pretreatment with only the lower dose of NBNI. Like β-FNA, NBNI pretreatment in the vlPAG at both doses prevented the expression of significant rppOFQ/$N_{135-151}$ antinociception elicited from the amygdala. In contrast, rats receiving NBNI pretreatment in misplaced mesencephalic sites displayed significant amygdala rppOFQ/$N_{135-151}$ antinociception with this response enhanced after 15-60 min.

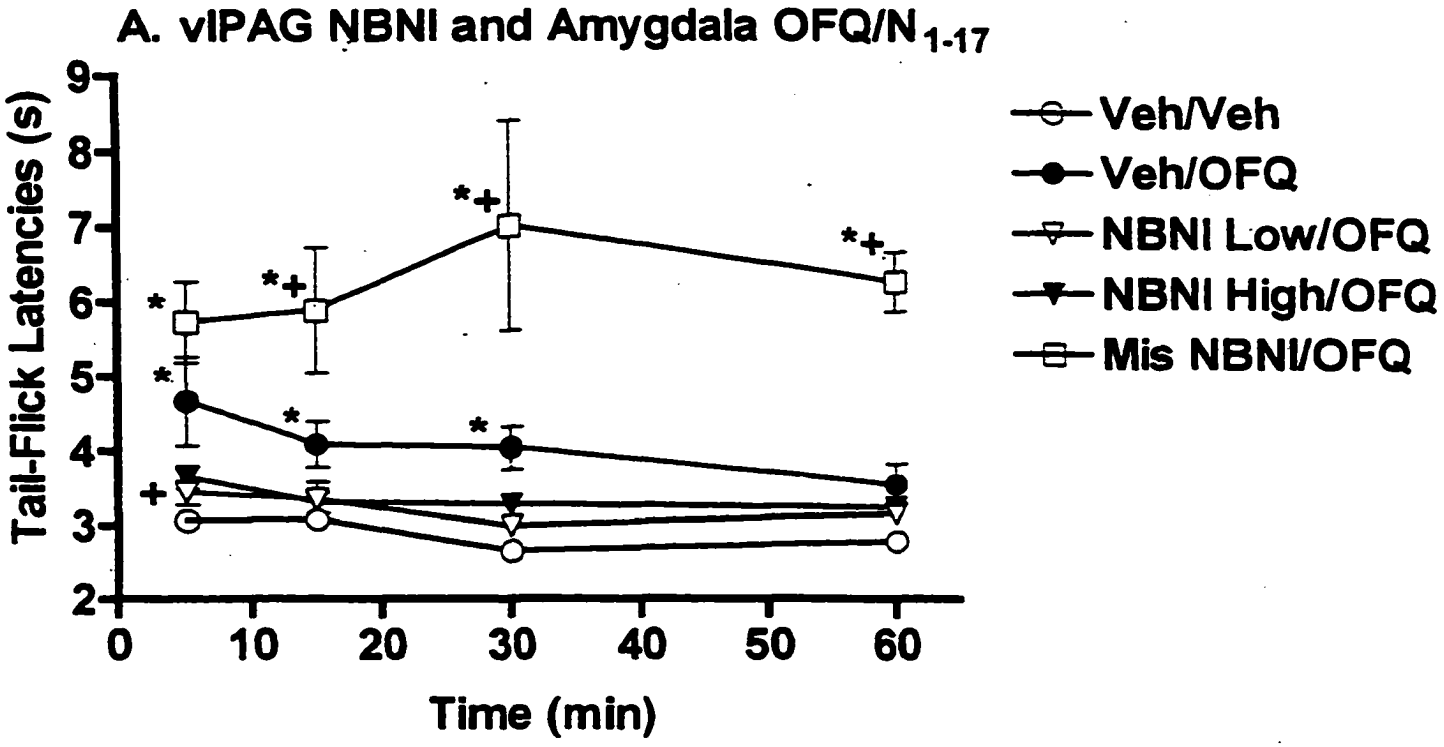
vlPAG NTI and rppOFQ/$N_{135-151}$ Antinociception elicited from theAmygdala:

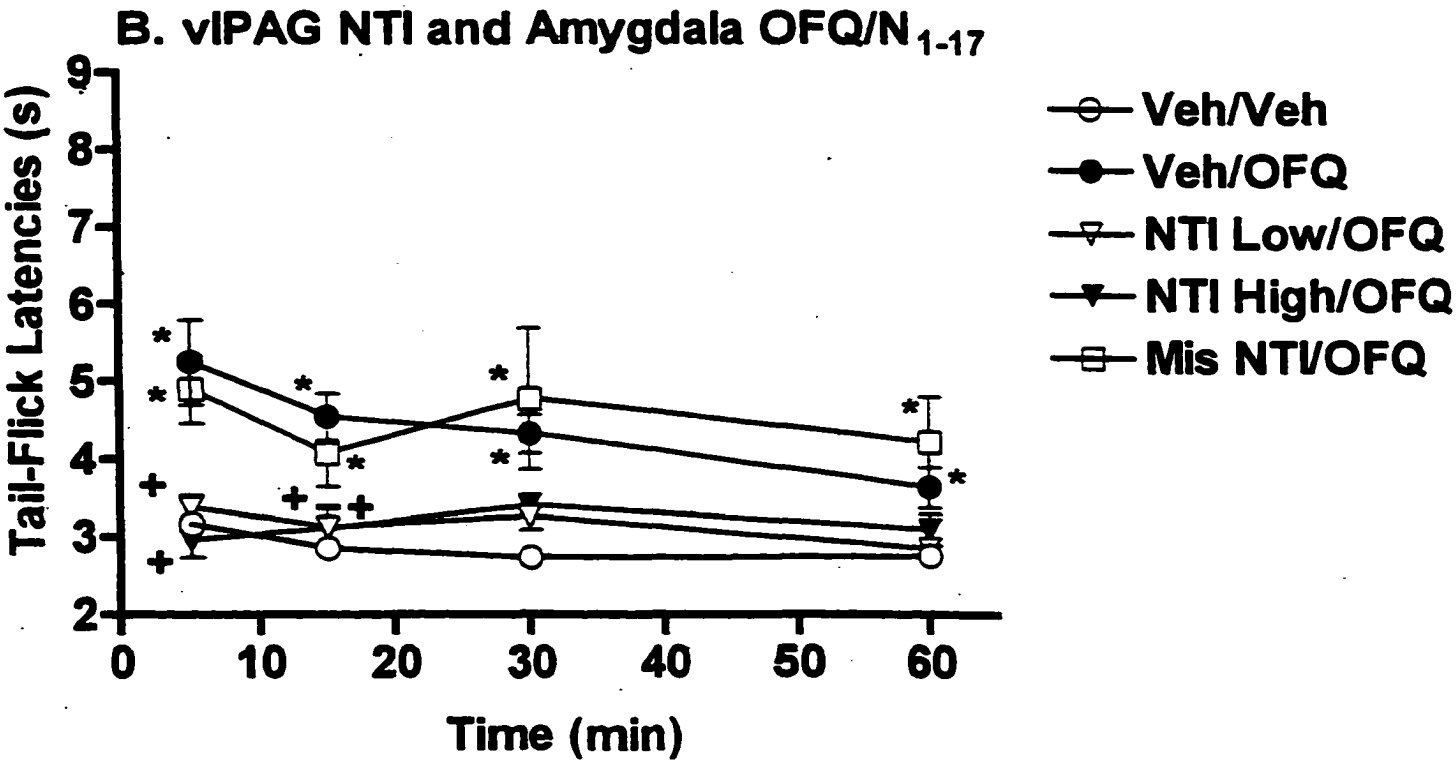
Significant differences were observed among experimental conditions ($F(4, 56) = 35.21$, $p<0.0001$), across test times ($F(3, 42) = 11.75$, $p<0.0001$) and for their interaction ($F(12, 168) = 3.63$, $p<0.0001$). rppOFQ/$N_{135-151}$ administered into the amygdala significantly increased latencies across the 60-min time course relative to vehicle treatment (Fig. 12, lower panel). Similar to naltrexone, the magnitude of rppOFQ/$N_{135-151}$ antinociception elicited from the amygdala was significantly reduced after 5 and 15 min following vlPAG pretreatment with either the lower or higher doses of NTI. In contrast, NTI pretreatment in misplaced mesencephalic sites failed to alter amygdala rppOFQ/$N_{135-151}$ antinociception.

rppOFQ/$N_{135-151}$ Antinociception elicited from the vlPAG and Opioid Antagonists in the Amygdala:

Significant differences were observed among experimental conditions ($F(5, 37) = 3.71$, $p<0.008$), across test times ($F(3, 15) = 2.86$, $p<0.04$), but not for their interaction

Figure 12. Alterations (Mean, ±SEM) in tail-flick latencies in rats receiving vehicle, rppOFQ/$N_{135-151}$ (5.5 μg) in the amygdala, or pretreatment with either nor-binaltorphamine (NBNI, Panel A) or naltrindole (NTI, Panel B) in the vlPAG prior to rppOFQ/$N_{135-151}$ into the amygdala of rats.





($F(15, 111) = 0.83$, n.s.). rppOFQ/$N_{135-151}$ administered into the vlPAG significantly increased latencies after 15, 30 and 60 min relative to vehicle treatment (Fig. 13). The magnitude of rppOFQ/$N_{135-151}$ antinociception elicited from the vlPAG was significantly reduced after 15, 30 and 60 min following amygdala pretreatment with naltrexone. However, amygdala pretreatment with an equimolar dose of NBNI significantly reduced rppOFQ/$N_{135-151}$ antinoccieption elicited from the vlPAG only after 15 min, but prevented its expression over the time course. In contrast, neither β-FNA nor NTI pretreatment in the amygdala significantly affected the magnitude of rppOFQ/$N_{135-151}$ antinociception elicited from the vlPAG.

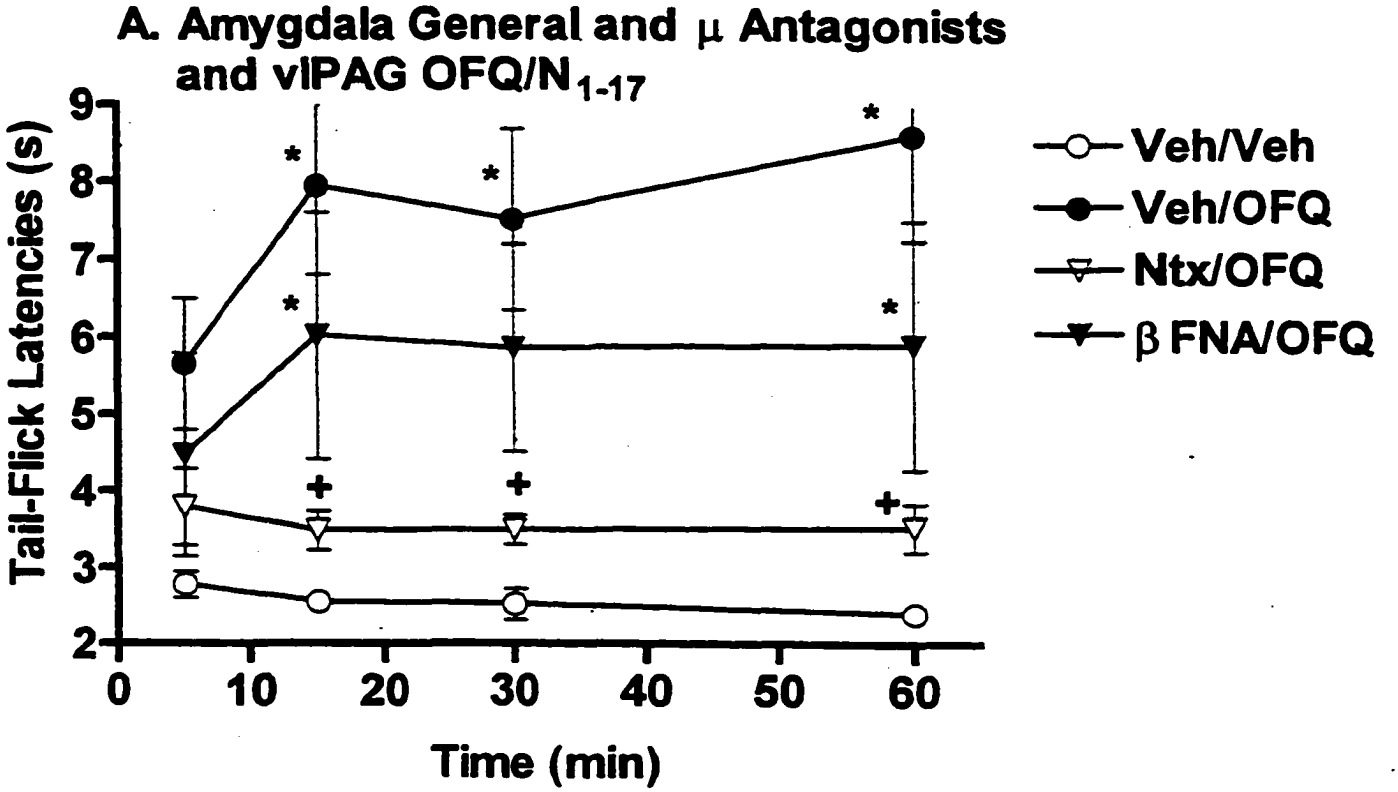
General Opioid Antagonism in the Amygdala and vlPAG:

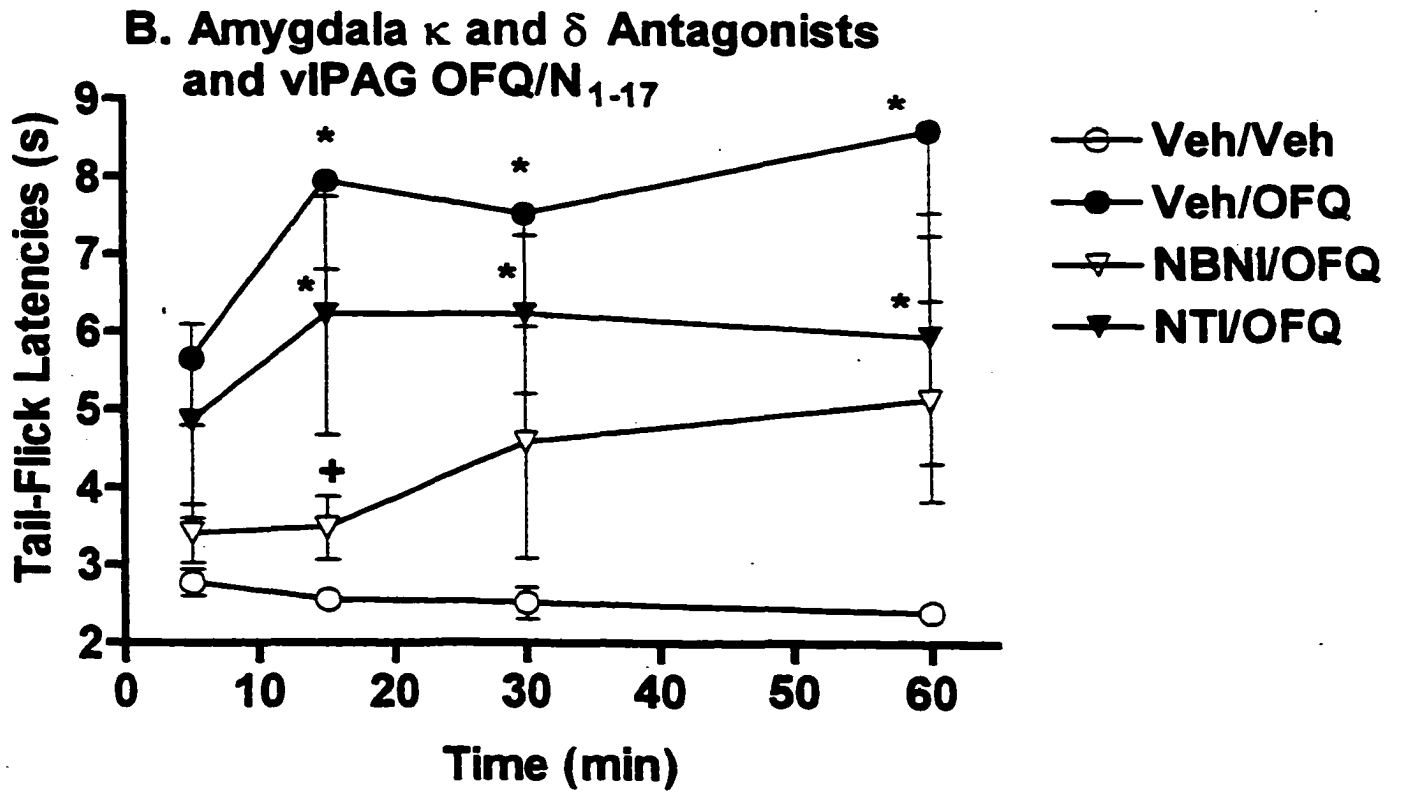
Significant differences were observed among experimental conditions ($F(2, 13) = 16.98$, $p<0.0002$), but not across test times ($F(3, 6) = 1.98$, n.s.) or for their interaction ($F(6, 39) = 1.29$, n.s.). Administration of the opioid antagonist, naltrexone into the amygdala produced small (~1.5 s), but significant increases in latencies after 25 and 55 min relative to vehicle treatment. In contrast, naltrexone administration into the vlPAG failed to alter latencies.

Discussion

The present study clearly indicated that rppOFQ/$N_{135-151}$-induced antinociception elicited from the amygdala is dependent upon an opioid synapse within the vlPAG for its full expression since pretreatment with the general opioid antagonist, naltrexone into the vlPAG significantly reduced the magnitude and duration of rppOFQ/$N_{135-151}$-induced antinociception elicited from the amygdala. The specificity of these effects was insured in two different ways. There was site-specificity since naltrexone administered into

Figure 13. Alterations (Mean, ±SEM) in tail-flick latencies in rats receiving vehicle, rppOFQ/$N_{135-151}$ (5.5 μg) in the vlPAG, or pretreatment with either naltrexone (NTX), β-funaltrexamine (β-FNA), nor-binaltorphamine (NBNI) or naltrindole (NTI) bilaterally in the amygdala prior to rppOFQ/$N_{135-151}$ into the vlPAG of rats.





mesencephalic sites lateral and ventral to the vlPAG failed to alter either the magnitude or the duration of rppOFQ/$N_{135-151}$-induced antinociception elicited from the amygdala. One alternative reason why naltrexone in the vlPAG could reduce rppOFQ/$N_{135-151}$-induced antinociception elicited from the amygdala is that the former injection could conceivably have elicited a hyperalgesic response that thereby cancelled the latter injection's antinociceptive response. The fact that naltrexone administration into the vlPAG alone failed to alter latencies discounts this alternative explanation. Therefore, it appears that an opioid synapse within the vlPAG is an integral part of the circuitry mediating rppOFQ/$N_{135-151}$-induced antinociception elicited from the amygdala. Therefore, the antinociceptive response to rppOFQ/$N_{135-151}$ elicited from the amygdala is quite similar in this respect to the antinociceptive responses of either DAMGO, morphine, β-endorphin or U50,488H elicited from the amygdala (Pavlovic et al., 1996x; Pavlovic and Bodnar, 1998x; Tershner and Helmstetter, 2000).

Opioid receptor subtype antagonist pretreatment into the vlPAG differentially altered rppOFQ/$N_{135-151}$-induced antinociception elicited from the amygdala such that the δ-selective opioid antagonist, NTI strongly and significantly reduced this antinociceptive response. In contrast, rppOFQ/$N_{135-151}$-induced antinociception elicited from the amygdala was marginally and transiently reduced by vlPAG pretreatment with either μ-selective (β-FNA) or κ-selective (NBNI) opioid receptor subtype antagonists. Again, each of these responses were site-specific since specific antagonist pretreatment in mesencephalic cannula placements ventral and lateral to the vlPAG failed to alter the magnitude or duration of rppOFQ/$N_{135-151}$-induced antinociception elicited from the amygdala. Thus, general and δ opioid receptor antagonists were more effective than μ

and κ opioid receptor antagonists in the vlPAG in mediating rppOFQ/$N_{135-151}$-induced antinociception elicited from the amygdala. This relationship is quite similar to the greater potency of general and δ relative to μ opioid receptor antagonists in the vlPAG to reduce amygdala antinociception following either morphine, β-endorphin or U50488H (Pavlovic et al., 1996x; Pavlovic and Bodnar, 1998). However, this pattern differs markedly from the approximately equal effectiveness of general, μ-selective, δ-selective and κ-selective opioid antagonist pretreatment within the amygdala upon subsequent rppOFQ/$N_{135-151}$-induced antinociception elicited from the amygdala (Chapter 5; Shane et al., 2002). The enhanced analgesia seen with the misplaced mesencephalic sites may be due to rppOFQ/$N_{135-151}$ activating a disinhibitory system somewhere downstream from the amygdala; however, based on the results of the present study, we do not know where this system is.

The present study also clearly indicated the existence of a reciprocal relationship in that rppOFQ/$N_{135-151}$-induced antinociception elicited from the vlPAG is dependent upon a bilateral opioid synapse within the amygdala for its full expression. This was supported by the finding that pretreatment with the general opioid antagonist, naltrexone into the amygdala significantly reduced the magnitude and duration of rppOFQ/$N_{135-151}$-induced antinociception elicited from the vlPAG. In assessing whether naltrexone in the amygdala produced a countervailing hyperalgesic response in and of itself, it was found that naltrexone treatment in the amygdala actually increased latencies, thereby eliminating this alternative explanation for the present effects. This small, but significant antinociception elicited by general opioid antagonism in the amygdala is in contrast to the inability of naltrexone to significantly alter latencies in other supraspinal sites like the

vlPAG or the RVM (e.g., Kiefel et al., 1993; Rossi et al., 1993, 1994). The receptor subtypes mediating this effect were not clearly defined using selective antagonists since pretreatment with either κ-selective (NBNI), μ-selective (β-FNA) or δ-selective (NTI) opioid antagonists were far less effective in significantly reducing rppOFQ/$N_{135-151}$-induced antinociception elicited from the vlPAG. The lesser effectiveness of NBNI and the ineffectiveness of either β-FNA or NTI administration into the amygdala upon rppOFQ/$N_{135-151}$-induced antinociception elicited from the vlPAG was not due to ineffective dose choices of the antagonists since each of the same antagonists administered into the amygdala potently reduced subsequent rppOFQ/$N_{135-151}$-induced antinociception elicited from the same nucleus.

These data provide further support to the hypothesis of both ascending and descending influences between supraspinal sites mediating pain-inhibition. Such sites as the amygdala, vlPAG and RVM elicit opioid-mediated antinociceptive responses that interact with each other in synergy studies (Pavlovic and Bodnar, 1998x; Rossi et al., 1993, 1994). A role for vlPAG and RVM synapses in mediating antinociceptive responses elicited by morphine, DAMGO, β-endorphin or U50488H administered into the amygdala has been conclusively established in antagonist studies (Helmstetter et al., 1999?; Pavlovic et al., 1996x; Pavlovic and Bodnar, 1998x; Tershner and Helmstetter, 2000). A role for RVM synapses in mediating antinociceptive responses elicited by morphine, but not β-endorphin administered into the vlPAG has also been conclusively established in antagonist studies (Kiefel et al., 1992a, 1992b, 1993; Spinella et al., 1996, 1997, 1999; Urban and Smith, 1993, 1994a, 1994b; vanPraag and Frenk, 1990). A role for ascending pain control systems from the vlPAG has been examined in a few studies.

Thus, naloxone pretreatment into the habenula significantly reduced vlPAG morphine-induced antinociception, and administration into the nucleus accumbens significantly reduced morphine-induced antinociception elicited from the habenula (Ma et al., 1992). Further, capsaicin-induced antinociception was significantly reduced by the GABA agonist, muscimol, but not the opioid antagonist, naloxone administered into the RVM, and was significantly reduced by either dopaminergic (flupentixol) or opioid (naloxone) antagonists administered into the nucleus accumbens (Gear et al., 1999). Finally, morphine antinociception elicited from the vlPAG is significantly reduced by either bilateral administration of the general serotonergic antagonist, methysergide into either the amygdala or the nucleus parafascicularis thalami or by unilateral methysergide administration into both the amygdala and nucleus parafascicularis thalami (Borszcz, 1999; Borszcz and Streltsov, 2000). The present findings that rppOFQ/$N_{135-151}$-induced antinociception elicited from the vlPAG is significantly reduced by general opioid antagonism pretreatment in the amygdala provide further support for the role of ascending influences in opioid pain-inhibitory systems.

CHAPTER 7: GENERAL DISCUSSION

Research, particularly over the past decade, has clearly established the amygdala as an important rostral brain site involved in pain inhibition. Thus, a number of opiate drugs, opioid peptides, opioid analogues, and endopeptidase inhibitors associated with the opioid system each are capable of eliciting antinociception from the amygdala. This dissertation clearly establishes that a number of peptide fragments of the ppOFQ/N precursor gene, which have been classically associated with paradoxical pro-nociceptive actions, each act selectively and specifically within the amygdala to produce antinociceptive responses. A relationship between classic opioid agonists and these ppOFQ/N gene peptides have also been established within the amygdala using opioid receptor subtype antagonists. This is supported by autoradiographic studies showing that the amygdala has an abundance of ORL-1 receptors (Letchworth et al., 2000) as well as other opioid receptor subtypes (Mansour, 1987). The amygdala does not stand alone in the modulation of these pain-inhibitory processes. Rather, it is clearly connected with other important parts of a supraspinal opioid pain-inhibitory system, particularly the vlPAG. Hence, the nuclei of the amygdala, known to elicit antinociceptive responses following administration of μ-receptor agonists morphine or DAMGO, β-endorphin, or the κ-selective agonist U50 488H, depend on an opioid synapse in the vlPAG for their full expression (Pavlovic et al., 1996a, 1996b, 1998; Helmstetter et al., 1998; Tershner and Helmstetter, 2000). Thus, pretreatment of the vlPAG with general and selective opioid receptor antagonists results in reduced opioid agonist-induced antinociception elicited from the amygdala. This dissertation confirms a similar relationship for

rppOFQ/$N_{135-151}$-induced antinociception elicited from the amygdala. Further, we have also established a role for the amygdala in mediating rppOFQ/$N_{135-151}$-induced antinociception elicited from the vlPAG. Finally, since the amygdala is also intimately involved in the mediation of stress-related responses (Goldstein et al., 1996; Walker et al., 1997), this makes it an ideal candidate for mediating the multi-faceted actions of rppOFQ/$N_{135-151}$.

Specific Aim One evaluated the antinociceptive and hyperalgesic effects of rppOFQ/$N_{135-151}$ and two of its truncated fragments rppOFQ/$N_{135-145}$ and OFQ/$N_{135-141}$ in the amygdala in rats on both radiant heat (tail-flick) and shock (jump threshold) tests. These fragments were chosen because rppOFQ/$N_{135-151}$ and its two active truncated versions, rppOFQ/$N_{135-145}$ and OFQ/$N_{135-141}$ were found to produce antinociception in mice following ventricular administration, which was blocked by antisense probes targeted at exons 2 and 3, but not exon 1 of the ORL-1 clone (Rossi et al., 1997). This study confirmed the antinociceptive actions of rppOFQ/$N_{135-151}$, and both active fragments, rppOFQ/$N_{135-145}$ and rppOFQ/$N_{135-141}$ after administration into the amygdala produce antinociception on the tail-flick test using high-intensity thermal stimuli set to produce short baseline latencies. In contrast, they each failed to alter jump thresholds when administered into the amygdala, indicating test specific antinociceptive responses of OFQ/N fragments in the amygdala for thermal, but not electrical noxious stimuli. This nociceptive test profile is quite different from other opioid agonists in the amygdala which show greater effectiveness on the jump test relative to the tail-flick test (Pavlovic and Bodnar, 1998; Pavlovic et al., 1996). These test-specific data suggest that the opioid receptors mediating traditional μ, κ and potentially δ agonists in the amygdala (Paden et

al., 1987) probably have a different distribution than OFQ/N binding sites identified in the amygdala using autoradiography (Letchworth et al., 2000).

The amygdala does not appear to support any of the hyperalgesic effects of rppOFQ/$N_{135-151}$ or its fragments at least under the dose and testing situations employed. The original reports that OFQ/N produced hyperalgesia (Kavaliers and Perrot-Sinalts, 1996; Meunier et al., 1995; Reinscheid et al., 1995) were subsequently modified (Meunier, 2000) such that rppOFQ/$N_{135-151}$ produced anti-opioid effects in both stress and opioid agonist antinociceptive assays (Grisel et al., 1996; Mogil et al., 1996a, 1996b, Tian et al., 1997). Given the selective deficits in opioid antinociceptive and opioid-mediated stress-induced analgesic responses following amygdala lesions (Fox and Sorenson, 1994; Helmstetter, 1993; Helmstetter et al., 1993; Manning and Mayer, 1995a; 1995b; Pavlovic et al., 1996a; Watkins et al., 1993), it would appear that this site would be a strong candidate for displaying such hyperalgesic responses. Yet neither rppOFQ/$N_{135-151}$, rppOFQ/$N_{135-145}$ nor rppOFQ/$N_{135-141}$ decreased low-intensity tail-flick latencies following amygdala microinjections; indeed, the trend of the data for each OFQ fragment indicated further increases in these longer latencies. One might question whether this is a reliable measure of such effects. First, this procedure yields a reliable hyperalgesia in the mouse following ventricular (Rossi et al., 1996), but not intrathecal (King et al., 1997) administration, suggesting a supraspinal mechanism of hyperalgesic action. Second, the profile of rppOFQ/$N_{135-151}$-induced hyperalgesia differs from that of rppOFQ/$N_{135-151}$-induced antinociception since antisense probes directed against coding exon 1, but not coding exons 2 and 3, of the ORL-1/KOR-3 clone block the former

response while antisense probes directed against coding exons 2 and 3, but not coding exon 1, block the latter response (Rossi et al., 1997).

These data raise the question as to whether the antinociceptive, but not hyperalgesic properties of rppOFQ/$N_{135-151}$ or its fragments administered into the amygdala are common to all ppOFQ/N fragments. This question was addressed in **Specific Aim Two.** The precursor responsible for producing rppOFQ/$N_{135-151}$ (Houtani et al., 1996; Meunier et al., 1995; Pan et al., 1996) yields OFQ/N2 (rppOFQ/$N_{154-170}$), which, in initial studies, were found to elicit similar antinociceptive effects to rppOFQ/$N_{135-151}$ when given i.c.v. in mice (Rossi et al., 1998). This study examined rppOFQ/$N_{154-181}$ in the amygdala as part of a larger study evaluating the effects of this peptide in other supraspinal sites as well including the vlPAG, RVM and LC. In analyzing the peptide fragments responsible for OFQ/N2-induced antinociception, we (Rossi et al., 2002) found that rppOFQ/$N_{154-181}$ produced antinociceptive *and* hyperalgesic responses using respective high-intensity and low-intensity tail-flick assays following microinjections into the locus coeruleus and RVM, but produces antinociceptive *without* hyperalgesic responses using these two respective procedures following microinjections into the vlPAG and amygdala.

Interestingly, in the four rat brain sites examined, rppOFQ/$N_{154-181}$ produced potent antinociception. However, the antinociceptive response in two of the cannulated sites (amygdala and PAG) was naloxone-reversible. Moreover, those sites that did not produce an opiate-reversible antinociception (the LC and the RVM), showed significant hyperalgesia. Therefore, these data indicate site-specificity mediating the differentiated mechanisms of antinociceptive actions and hyperalgesia. The presence of this double-

dissociation serves as an important control in analyzing the specificity of the responses. Previous research shows that pronociceptive and antinociceptive effects of OFQ/N are dissociable. The first line of evidence comes from earlier studies showing that antisense which targets the coding region of exon 1 of the ORL1 receptor clone decreases OFQ/N hyperalgesia when given icv, however, antisense targeting exons 2 and 3 of the same clone are ineffective icv. In contrast, ORL1 antisense to exons 2 and 3 decrease OFQ/N icv analgesia, while ORL1 exon 1 is ineffective in the RVM and LC (Rossi et al., 1997). In addition, other laboratories have confirmed the possibility of dissociation in a single brain region. Fields and his co-workers demonstrated ON-OFF cells in the RVM which were both pain-facilitory and pain-inhibitory systems (Fields et al., 1991). Therefore, the present study demonstrates that it is possible to produce both pronociceptive and antinociceptive effects following rppOFQ/$N_{154-181}$ microinjection. Moreover, this possibility of opiate or non-opiate action is clearly based upon the supraspinal site of action. The ability of the full-length peptide, rppOFQ/$N_{154-181}$ to induce hyperalgesia is site-specific with caudal sites (LC and RVM) mediating this response. These groups of sites behave quite similarly in eliciting traditional opioid antinociception with greatest sensitivity to mu-selective ligands (Bodnar et al., 1988, Smith et al., 1988, Pavlovic et al., 1996, Fang et al., 1986, Helmstetter et al., 1995) as well as exhibiting antinociceptive opioid synergy using morphine and β-endorphin (Rossi et al., 1993, 1994; Pavlovic and Bodnar, 1998).

Interestingly, this suggests that different functional and physiological mechanisms other than the amygdala and PAG may mediate the pro-nociceptive effects of the full-length peptide in the rostral part of the brain. In addition, previous studies have shown a

dissociation between spinal *versus* supraspinal sites of action for OFQ/N. Supraspinally-administered OFQ/N was capable of producing both hyperalgesia and antinociception, whereas spinally-administered OFQ/N produced a robust antinociception that was naloxone-sensitive, but failed to produce hyperalgesia (King et al., 1997). These, and other, studies provide insight into the underlying mechanisms by which novel, and often divergent, physiological functions arise in opioid and non-opioid systems (Danielson, et al., 2001). Therefore, it is both possible and probable, that the OFQ/N system may have evolved as a separate and distinct system for pain perception.

Thus, this related OFQ/N peptide seems to share very similar characteristics relative to rppOFQ/$N_{135-151}$ and its truncated fragments. This suggests that perhaps all of the biologically-active fragments of ppOFQ/N may act similarly with respect to modulating antinociceptive and pro-nociceptive actions. This still however doesn't answer the question about potential relationships between ppOFQ/N peptides and traditional opioid receptor systems.

Thus, **Specific Aim Three** determined that antinociception elicited by rppOFQ/$N_{135-151}$ administered in the amygdala is dependent upon the intact functioning of μ, κ and δ opioid receptors in the amygdala since pretreatment with either general or selective opioid antagonists in the amygdala significantly reduced this agonist's antinociceptive response. Autoradiographic and immunihistochemical techniques have shown that there is a dense distribution of ORL-1 and traditional opioid receptors in the amygdala (Letchworth et al., 2000; Mansour et al., 1987, 1994a, 1994b, 1994c, 1995a, 1995b, 1996). Antinociception elicited following amygdala microinjections of the selective κ_1 agonists U50 488H is blocked by amygdala pretreatment of general and

selective κ_1 opioid antagonists. It is unlikely that OFQ/N_{1-17} is binding directly with μ, κ or δ receptors in the amygdala (Mansour et al., 1987; 1994a; 1994b; 1994c; 1995a; 1995b; 1996) to elicit analgesic effects rather than to the ORL-1/KOR-3 receptor in the amygdala (Anton et al., 1996; Letchworth et al., 2000; Neal et al., 1999a) since definitive initial characterization of rppOFQ/$N_{135-151}$ showed that it binds poorly to traditional μ, κ and δ opioid receptors as compared to its high affinity for ORL-1/KOR-3 receptors (Butour et al., 1997; Meunier et al., 1995; Reinscheid et al., 1995). It is also improbable that the four antagonists (NTX, β-FNA, NBNI, NTI) are successfully competing with rppOFQ/$N_{135-151}$ in the amygdala to act at the ORL-1/KOR-3 receptor rather than acting at their respective traditional opioid receptor subtypes given the high affinity of rppOFQ/$N_{135-151}$ for the ORL-1/KOR-3 receptor (Butour et al., 1997; Meunier et al., 1995; Reinscheid et al., 1995) relative to the poor and even negligible affinity for the ORL-1/KOR-3 receptor of traditional μ (e.g., morphine, fentanyl) κ (dynorphin A_{1-8}, tifluadom) or δ (e.g., D-Pen^2, D-Pen^5-enkephalin) agonists which are readily and respectively reversed by β-FNA, NBNI and NTI (Bunzow et al., 1994; Mollereau et al., 1994, Pan et al., 1994; Pan et al., 1995; Wick et al., 1994). Studies have revealed that the medial amygdaloid nucleus is a common site at which dense distributions of traditional μ, κ and δ opioid receptors. However, the autoradiographic and mRNA techniques used to visualize these receptors in the amygdala do not specify their synaptic distribution so it is not clear whether such interactions occur at the same neuron or at trans-synaptically linked neurons. Nevertheless, the present evidence strongly suggests that rppOFQ/$N_{135-151}$ produces antinociception elicited from the amygdala by activating traditional opioid

receptor synapses within the same structure. This then led us into a last series of studies linking the amygdala with a prototypical traditional pain-inhibitory nucleus, the vlPAG.

Specific Aim Four clearly indicated that rppOFQ/$N_{135-151}$-induced antinociception elicited from the amygdala is dependent upon an opioid synapse within the vlPAG for its full expression since pretreatment with the general opioid antagonist, naltrexone into the vlPAG significantly reduced the magnitude and duration of rppOFQ/$N_{135-1517}$-induced antinociception elicited from the amygdala.

Antinociceptive responses following amygdala microinjections of traditional opioid agonists such as morphine, DAMGO, β-endorphin and U50 488H have been linked to an intrinsic pain-inhibitory pathway originating in the vlPAG. (Krettak and Price, 1978; Beitz, 1982; Basbaum and Fields, 1984; Rizvy et al., 1991). Pretreatment of the vlPAG with lidocaine or microinjections of either general or selective μ or δ_2 opioid receptor antagonists significantly reduced the antinociception elicited by either morphine, β-endorphin, DAMGO or U50 488H in the amygdala (Pavlovic et al., 1996; Pavlovic et al., 1998; Helmstetter et al., 1998; Tershner and Helmstetter, 2000).

Opioid receptor subtype antagonist pretreatment into the vlPAG differentially altered rppOFQ/$N_{135-151}$-induced antinociception elicited from the amygdala in that general and δ opioid receptor antagonists were more effective than μ and κ opioid receptor antagonists in the vlPAG in mediating rppOFQ/$N_{135-151}$-induced antinociception elicited from the amygdala. This relationship is quite similar to the greater potency of general and δ relative to μ opioid receptor antagonists in the vlPAG to reduce amygdala antinociception following either morphine, β-endorphin or U50488H (Pavlovic et al., 1996x; Pavlovic and Bodnar, 1998). However, this pattern differs markedly from the

approximately equal effectiveness of general, μ-selective, δ-selective and κ-selective opioid antagonist pretreatment within the amygdala upon subsequent rppOFQ/$N_{135-151}$-induced antinociception elicited from the amygdala (Chapter 5; Shane et al., 2002).

The existence of a reciprocal relationship in that rppOFQ/$N_{135-151}$-induced antinociception elicited from the vlPAG is dependent upon a bilateral opioid synapse within the amygdala for its full expression was also indicated in **Specific Aim Four**. This was supported by the finding that pretreatment with the general opioid antagonist, naltrexone into the amygdala significantly reduced the magnitude and duration of rppOFQ/$N_{135-151}$-induced antinociception elicited from the vlPAG. The receptor subtypes mediating this effect were not clearly defined using selective antagonists since pretreatment with either κ-selective (NBNI), μ-selective (β-FNA) or δ-selective (NTI) opioid antagonists were far less effective in significantly reducing rppOFQ/$N_{135-151}$-induced antinociception elicited from the vlPAG.

These data provide further support to the hypothesis of both ascending and descending influences between supraspinal sites mediating pain-inhibition. Such sites as the amygdala, vlPAG and RVM elicit opioid-mediated antinociceptive responses that interact with each other in synergy studies (Pavlovic and Bodnar, 1998x; Rossi et al., 1993, 1994). A role for vlPAG and RVM synapses in mediating antinociceptive responses elicited by morphine, DAMGO, β-endorphin or U50488H administered into the amygdala has been conclusively established in antagonist studies (Helmstetter et al., 1999?; Pavlovic et al., 1996x; Pavlovic and Bodnar, 1998x; Tershner and Helmstetter, 2000). A role for RVM synapses in mediating antinociceptive responses elicited by morphine, but not β-endorphin administered into the vlPAG has also been conclusively

established in antagonist studies (Kiefel et al., 1992a, 1992b, 1993; Spinella et al., 1996, 1997, 1999; Urban and Smith, 1993, 1994a, 1994b; vanPraag and Frenk, 1990). A role for ascending pain control systems from the vlPAG has been examined in a few studies. Thus, naloxone pretreatment into the habenula significantly reduced vlPAG morphine-induced antinociception, and administration into the nucleus accumbens significantly reduced morphine-induced antinociception elicited from the habenula (Ma et al., 1992). Further, capsaicin-induced antinociception was significantly reduced by the GABA agonist, muscimol, but not the opioid antagonist, naloxone administered into the RVM, and was significantly reduced by either dopaminergic (flupentixol) or opioid (naloxone) antagonists administered into the nucleus accumbens (Gear et al., 1999). Finally, morphine antinociception elicited from the vlPAG is significantly reduced by either bilateral administration of the general serotonergic antagonist, methysergide into either the amygdala or the nucleus parafascicularis thalami or by unilateral methysergide administration into both the amygdala and nucleus parafascicularis thalami (Borszcz, 1999; Borszcz and Streltsov, 2000). The present findings that rppOFQ/$N_{135-151}$-induced antinociception elicited from the vlPAG is significantly reduced by general opioid antagonism pretreatment in the amygdala provide further support for the role of ascending influences in opioid pain-inhibitory systems.

The findings presented in this dissertation have provided a more detailed understanding of the receptor pharmacology and antinociceptive actions of fragments of the ppOrph gene. Several studies support the antinociceptive actions of OFQ/N, and the mediation of these effects through the utilization of an opioid system (Rossi et al., 2002; Gintzler et al., 1997; Shane et al., 2002, in press). The endogenous opioid system

modulates a number of physiological functions and behaviors including gastrointestinal motility, respiration, feeding, stress, and antinociception. A main conclusion of these studies is that the role of OFQ/N in the modulation of these behaviors can be considered as part of a larger physiological system that is mediated by other traditional opioids and has evolved to maintain homeostatic functions rather than the inherently "different" profile ascribed to OFQ/N in its initial characterization (Meunier et al., 1995; Reinscheid et al., 1995).

References

Abols, I.A., & Basbaum, A.I. (1981) Afferent connections of the rostral medulla of the cat: a neural substrate for midbrain-medullary interactions. Journal of Comparative Neurology, 201, 285-297.

Adler, M.W., & Geller, E.B. (1993) Physiological functions of opioids: Temperature regulation. . Handbook of Experimental Pharmacology, Opioids II. Springer-Verlag:Berlin. 205-229.

Akil, H., Watson, S.J., Young, E., Lewis, M.E., Khachaturian, H., & Walker, J.M. (1984) Endogenous opioids: biology and function. Annual Review of Neuroscience, 7, 223-255.

Al-Rodhan, N., Chipkin, R., & Yaksh, T.L. (1990) The antinociceptive effects of SCH-32615, a neutral endopeptidase (enkephalinase) inhibitor, microinjected into the periaqueductal, ventral medulla and amygdala. Brain Research, 520, 123-130.

Anton, B., Fein, J., To, T., Li, X., Silberstein, L., & Evans, C.J. (1996) Immunohistochemical localization of ORL-1 in the central nervous system of the rat. Journal of Comparative Neurology, 368, 229-251.

Archer, S. (1993) Chemistry of non-peptide opioids. . Handbook of Experimental Pharmacology, Opioids I. Springer-Verlag:Berlin. 241-271.

Basbaum, A.I., & Fields, H.L. (1984) Endogenous pain control systems: brainstem spinal pathways and endorphin circuitry. Annual Review of Neuroscience, 7, 309-338.

Beitz, A.J. (1982) The sites of origin of brainstem neurotensin and serotonin projections to the rodent nucleus raphe magnus. The Journal of Neuroscience, 2, 829-842.

Beitz, A.J., Mullett, M.A., & Weiner, L.L. (1983) The periaqueductal gray projections to the rat spinal trigeminal, raphe magnus, gigantocellualr pars alpha, and paragigantocellular nuclei arise from separate neurons. Brain Research, 288, 307-314.

Bellgowan, P.S., & Helmstetter, F.J. (1996) Neural systems for the expression of hypoalagesia during nonassociative fear. Behavioral Neuroscience, 110, 727-736.

Bennet, G.J., Abdelmoumene, M., Hayashi, H., & Dubner, R. (1980) Physiology and morphology of substantia gelatinosa neurons intracellularly stained with horseradish peroxidase. Journal of Comparative Neurology, 194, 809-827.

Bodnar, R.J. (1996) Opioid receptor subtype antagonists and ingestion. Cooper, S.J., & Clifton, P.J. Drug receptor subtypes and ingestive behaviour. Academic Press:London. 127-146.

Bodnar, R.J. (2000) Supraspinal circuitry mediating opioid antinociception: antagonist and synergy studies in multiple sites. Journal of Biomedical Sciences, 7, 181-194.

Bodnar, R.J., Paul, D., & Pasternak, G.W. (1991) Synergistic analgesic interactions between the periaqueductal gray and the locus coeruleus: studies with the partial mu-1 agonist ethylketocyclazocine. Brain Research, 558, 224-230.

Bodnar, R.J., Williams, C.L., Lee, S.J., & Pasternak, G.W. (1988) Role of mu1 opiate receptors in supraspinal opiate analgesia: a microinjection study. Brain Research, 447, 25-34.

Bohus, B., Koolhaas, J.M., Korte, S.M., Roozendaal, B., & Wiersma, A. (1996) Forebrain pathways and their behavioral interactions with neuroendocrine and cardiovascular function in the rat. Clinical Experimental Pharmacology and Physiology, 23(2), 177-182.

Boom, A., Mollereau, C., & Meunier, J.C. (1999) Distribution of the nociceptin and nocistatin precursor transcript in the mouse central nervous system. Neuroscience, 91, 991-1007.

Borszcz, G.C., & Streltsov, N.G. (2000) Amygdaloid-thalamic interactions mediate the antinociceptive action of morphine microinjected into the periaqueductal gray. Behavioral Neuroscience, 114, 574-584.

Borszcz, G.S. (1999) Differential contributions of medullary, thalamic and amygdaloid serotonin to the antinociceptive action of morphine administered into the periquesductal gray: A model of morphine analgesia. Behavioral Neuroscience, 113, 612-631.

Bowen, W.D., Hellewell, S.B., Kelemen, M., Huey, R., & Steward, D. (1987) Affinity labelling of delta-opiate receptors using [D-Ala-2,Leu-5,Cys-6]-enkephalin: covalent attachment via thiosulfide exchange. Journal of Biological Chemistry, 262, 13434-13439.

Brodie, M.S., & Proudfit, H.K. (1984) Hypoalgesia induced by the local injection of carbachol into the nucleus raphe magnus. Brain Research, 291, 337-342.

Brownstien, M.J. (1980) Opioid peptides: Search for the precursors. Nature, 287, 678-679.

Bunzow, J.R., Saez, C., Mortrud, M., Bouvier, C., Williams, J.T., Low, M., & Grandy, D.K. (1994) Molecular cloning and tissue distribution of a putative member of the rat opioid receptor gene family that is not a mu, delta or kappa receptor type. FEBS Letters, 347, 284-288.

Butour, J.L., Moisand, C., Mazarguil, H., Mollereau, C., & Meunier, J.C. (1997) Recognition and activation of the opioid receptor-like ORL 1 receptor by nociceptin, nociceptin analogues and opioids. European Journal of Pharmacology, 321, 97-103.

Cartsens, E. (1986) Hypothalamic inhibition of rat dorsal horn neuronal responses to noxious skin heating. Pain, 25, 95-107.

Castiglioni, A., Gallaway, M., & Coulter, J. (1978) Spinal projections from the midbrain in monkey. Journal of Comparative Neurology, 178, 329-346.

Chance, W.T. (1980) Autoanalgesia: opiate and non-opiate mechanisms. Neuroscience and Biobehavioral Reviews, 4, 55-67.

Chavkin, C., James, I.F., & Goldstein, A. (1982) Dynorphin is a specific endogenous ligand of the kappa receptor. Science, 215, 413-415.

Chen, Y., Fan, Y., Liu, J., Mestek, A., Tian, M., Kozak, C.A., & Yu, L. (1994) Molecular cloning, tissue distribution and chromosomal localization of a novel member of the opioid receptor gene family. FEBS Letters, 347, 279-283.

Chu, T.G., & Orlowski, M. (1985) Soluble metalloendopeptidase from rat brain: action on enkephalin-containing peptides and other bioactive peptides. Endocrinology, 116, 1418-1425.

Ciccocioppo, R., Panocka, I., Polidori, C., Regoli, D., & Massi, M. (1999) Effect of nociceptin on alcohol intake in alcohol-preferring rats. Psychopharmacology, 141, 220-224.

Ciccocioppo, R., Angeletti, S., Panocka, I., & Massi, M. (2000). Nociceptin/Orphanin FQ and drugs of abuse. Peptides, 21, 1071-1080.

Clark, J.A., Liu, L., Price, M., Hersh, B., Edelson, M., & Pasternak, G.W. (1989) Kappa opiate receptor multiplicity: evidence for two U50,488H-sensitive K-1 subtypes and a novel K-3 subtype. Journal of Pharmacology and Experimental Therapeutics, 251, 461-468.

Cotton, R., Giles, M.G., Miller, L., Shaw, J.S., & Timms, D. (1984) ICI 174864: a highly selective antagonist for the opioid delta receptor. European Journal of Pharmacology, 97, 331-332.

Cowan. (1993) Effects of opioids on the spontaneous behavior of animals. . Handbook of Experimental Pharmacology, Opioids II. Springer-Verlag:Berlin. 393-414.

Crisp, T., Smith, D.J., Perrotti, J.M., & Amedro, J.B. (1986) The antinociceptive action of intrathecal morphine involves the activation of a local seratonergic component in the spinal cord. Society for Neuroscience Abstracts, 12, 619.

Danielson, P.B., Hoverston, M.T., Fitzpatrick, M., Schreck, C., Akil, G., & Dores, R.M. (2001) Sturgeon orphanon A molecular "fossil" that bridges the gap between the opioids and orphanin FQ/N. Journal of Biological Chemistry, 276, 22114-22119.

Davis, M. (1992) The role of the amygdala in conditioned fear. Aggleton, J.P. The Amygdala. Wiley-Liss:New York. 255-306.

Dawson-Basoa, M., & Gintzler, A.R. (1997) Nociceptin (Orphanin FQ) abolishes gestational and ovarian sex steroid-induced antinociception and induces hyperalgesia. Brain Research, 750, 48-52.

Day, R., Trujillo, K.A., & Akil, A. (1993) Prodynorphin biosynthesis and posttranslational processing. . Handbook of Experimental Pharmacology, Opioids I. Springer-Verlag:Berlin. 449-463.

de Olmos, J., Alheid, G.F., & Beltramino, C. (1985) Amygdala. Paxinos, G. The rat nervous system I. Forebrain and midbrain. Academic Press:New York. .

Devine, D.P., Reinscheid, R.K., Monsma, F.J., Civelli, O., & Akil, H. (1996) The novel neuropeptide orphanin FQ fails to produce conditioned place preference or aversion. Brain Research, 727, 225-229.

DiChiara, G.D., & North, R.A. (1992) Neurobiology of opiate abuse. Trends in Pharmacological Sciences, 13, 185-193.

Evans, C., Keith, D., Morrison, H., Magendzo, K., & Edwards, R. (1992) Cloning of a delta-opioid receptor by functional expression. Science, 258, 1952-1955.

Fang, F.G., Fields, H.L., & Lee, N.M. (1986) Action at the mu receptor is sufficient to explain supraspinal analgesic effect of opiates. Journal of Pharmacology and Experimental Therapeutics, 238, 1039-1044.

Fields, H.L. (1993) Brainstem mechanisms of pain modulation: anatomy and physiology. In Herz, A. Handbook of Experimental Pharmacology, Opioids II. Springer-Verlag:Berlin. 3-15.

Fields, H., & Basbaum, A.I. (1978) Brain control of spinal pain transmission neurons. Annual Review of Physiology, 40, 217-248.

Fields, H.L., Heinricher, M.M., & Mason, P. (1991) Neurotransmitters in nociceptive modulatory circuits. Annual Review of Neuroscience, 14, 219-245.

Fleetwood-Walker, S.M., Mitchell, R., Hope, P.J., Molony, V., & Iggo, A. (1985) An alpha2 receptor mediates the selective inhibition by noradrenaline of nociceptive respnses of identified dorsal horn neurons. Brain Research, 334, 243-254.

Florin, S., Suaudeau, C., Meunier, J.C., & Costentin, J. (1996) Nociceptin stimulates locomotion and exploratory behavior in mice. European Journal of Pharmacology, 317, 9-13.

Fox, R.J., & Sorenson, C.A. (1994) Bilateral lesions of the amygdala attenuate analgesia induced by diverse environmental challenges. Brain Research, 648, 215-221.

Gear, R.W., Miaskowski, C., Gordon, N.C., Paul, S.M., Heller, P.H., & Levine, J.D. (1999) The kappa opioid nalbuphine produces gender-and dose-dependent analgesia and antianlgesia in patients with postoperative pain. Pain, 83, 339-345.

Gibson, M.J., Liotta, A.S., & Krieger, D.T. (1981) The Zucker fa/fa rat: absent circadian corticosterone periodicity and elevated beta-endorphin concentrations in brain and neurointermediate pituitary. Neuropeptides, 1, 349-362.

Gintzler, A.R., Adapa, I.D., Toll, L., Medina, V.M., & Wang, L. (1997) Modulation of enkephalin release by nociceptin (orphanin FQ). European Journal of Pharmacology, 325, 29-34.

Gistrak, M.A., Paul, D., Hahn, E.F., & Pasternak, G.W. (1989) Pharmacological actions of a novel mixed opiate agonist-antagonist: naloxone benzoylhydrazone. Journal of Pharmacology and Experimental Therapeutics, 251, 469-476.

Goldstein, A., Fischli, W., Lowney, L.I., Hunkapiller, M., & Hood, L. (1981) Porcine pituitary dynorphin: complete amino acid sequence of the biologically active heptadecapeptide. Proceedings of the National Academy of Science (USA), 74, 7219-23.

Goldstein, A., & Naidu, A. (1989) Multiple opioid receptors: Ligand selectivity profiles and binding signatures. Molecular Pharmacology, 36, 265-272.

Goldstein, A., Tachibana, S., Lowney, L.I., Hunkapiller, M., & Hood, L. (1979) Dynorphin(1-13), an extroadinarily potent opioid peptide. Proceedings of the National Academy of Science (USA), 76, 6666-6670.

Goldstein, L.E., Rasmusson, A.M., Bunney, B.S., & Roth, R.H. (1996) Role of the amygdala in the coordination of behavioral, neuroendocrine, and prefrontal cortical monoamine responses to psychological stress in the rat. The Journal of Neuroscience, 16(5), 4787-4798.

Goodman, R.R., & Pasternak, G.W. (1985) Visualization of mu-1 opiate receptors in rat brain using a computerized autoradiographic subtraction technique. Proceedings of the National Academy of Science (USA), 82, 6667-71.

Gray, T.S., & Bingaman, E.W. (1996) The amygdala: corticotropin-releasing factor, steroids, and stress. Critical Reviews in Neurobiology, 10(2), 155-168.

Gray, T.S., Carney, M.E., & Magnusen, D.J. (1989) Direct projections from the central amygdaloid nucleus to the hypothalamic paraventricular nucleus:possible role in stress-induced adrenocorticotropin release. Neuroendocrinology, 50, 433-446.

Grisel, J.E., Mogil, J.S., Belknap, J.K., & Grandy, D.K. (1996) Orphanin FQ acts as a supraspinal, but not a spinal, anti-opioid peptide. Neuroreport, 7, 2125-2129.

Guillemin, R., Ling, N., & Burgus, R. (1976) Endorphins, peptides d'origine hypothalmique et neurohypophsaire d'activate morphinomimetique. Isolement et structure moleculaire d'alpha-endorphin. Competes Rendus Academe des Sciences Series D, 282, 783-785.

Gupta, D.S., Kelson, A.B., Polgar, W.E., Tol, L., Szucs, M., & Gintzler, A.R. (2001) Ovarian sex steroid-dependent plasticity of nocicpetin/orphanin FQ and opioid modulation of spinal dynorphin release. Journal of Pharmacology and Experimental Therapeutics, 298(3), 1213-1220.

Hara, N., Minami, T., & Okuda-Ashitaka, E. (1997) Characterization of nociceptin hyperalgesia and allodynia in conscious mice. British Journal of Pharmacology, 121, 401-408.

Harrison, L.M., & Grady, D.K. (2000) Opiate modulating properies of nociceptin/orphanin FQ. Peptides, 21, 151-172.

Heinricher, M.M., McGaraughty, S., & Farr, D.A. (1999) The role of excitatory amino acid transmission within the rostral ventromedial medulla in the antinociceptive actions of systemically administered morphine. Pain, 81, 57-65.

Heinricher, M.M., & Roychowdhury, S.M. (1997) Reflex-related activation of putative pain facilitating neurons in rostral ventromedial medulla requires excitatory amino acid transmission. Neuroscience, 78, 1159-1165.

Helmstetter, F.J. (1992) The amygdala is essential for the expression of conditional hypoalgesia. Behavioral Neuroscience, 106, 518-528.

Helmstetter, F.J. (1993) Stress-induced hypoalgesia and defensive freezing are attenuated by application of diazepam to the amygdala. Pharmacology Biochemistry and Behavior, 44, 433-438.

Helmstetter, F.J., & Bellgowan, P.S. (1993) Lesions of the amygdala block conditional hypoalgesia on the tail-flick test. Brain Research, 612, 253-257.

Helmstetter, F.J., Bellgowan, P.S., & Tershner, S.A. (1993) Inhibition of the tail-flick reflex following microinjection of morphine into the amygdala. Neuroreport, 4, 471-474.

Helmstetter, F.J., Tershner, S.A., Poore, L.H., & Bellgowan, P.S.F. (1998) Antinociception following opioid stimulation of the basolateral amygdala is expressed

through the periaqueductal gray and rostral ventromedial medulla. Brain Research, 779, 104-108.

Herkenham, M., Moon Edley, S., & Stuart, J. (1984) Cell clusters in the nucleus accumbens of the rat, and the mosaic relationship of opiate receptors, acetylcholinesterase and subcortical afferent terminations. Neuroscience, 11, 561-593.

Heyman, J.S., Mulvaney, H.I., Mosberg, H.I., & Porreca, F. (1987) Opioid delta receptor involvement in supraspinal and spinal antinociception in mice. Brain Research, 420, 100.

Heyman, J.S., Vaught, J.L., Raffa, R.B., & Porreca, F. (1988) Can supraspinal delta opioid receptors mediate antinociception? Trends in Pharmacological Sciences, 9, 134-138.

Hollt, V. (1986) Opioid peptide processing and receptor selectivity. Annual Reviews of Pharmacology and Toxicology, 26, 59-77.

Houde, R.W., & Wallenstein, S.L. (1956) Clinical studies of morphine-nalorphine combinations. Federation Proceedings, 15, 440-441.

Houtani, T., Nishi, M., Takeshima, H., Nukada, T., & Sugimoto, T. (1996) Structure and regional distribution of nociceptin/orphanin FQ precursor. Biochemical and Biophysical Research Communications, 219, 714-719.

Howe, J.R., Wang, J.-Y., & Yaksh, T.L. (1983) Selective antagonism of the antinociceptive effect of intrathecally applied alpha adrenergic agonists by intrathecal prazosin and intrathecal clonidine. Journal of Pharmacology and Experimental Therapeutics, 224, 552-558.

Hughes, J., Smith, T., Kosterlitz, H.W., Fothergill, L.A., Morgan, B.A., & Morris, H.R. (1975) Identification of two related penta-peptides from the brain with potent opiate agonist activity. Nature, 258, 577-579.

Hunt, S.P., Kelly, J.S., & Emson, P.C. (1980) The electron microscopic localization of methionine-enkephalin within the superficial layers (I and II) of the spinal cord. Neuroscience, 5, 1871-1890.

Iwamoto, E.T. (1989) Antinociception after nicotine administration into the mesopontine tegmentum of rats: evidence for muscarinic actions. Journal of Pharmacology and Experimental Therapeutics, 251, 412-421.

Jenck, F., Moreau, J.L., Martin, J.R., Kilpatrick, G.J., Reinscheid, R.K., Monsma, F.J., Nothacker, H.P., & Civelli, O. (1997) Orphanin FQ acts as an anxiolytic to attenuate behavioral responses to stress. Proceedings of the National Academy of Science (USA), 94, 14854-14858.

Jensen, T.S., & Yaksh, T.L. (1986) III. Comparison of antinociceptive action of mu and delta opioid receptor ligands in the periaqueductal gray matter, medial and paramedial ventral medulla in the rat as studied by the microinjection technique. Brain Research, 372, 301-312.

Jhamandas, K.H., Sutak, M., & Henderson, G. (1998) Antinociceptive and morphine modulatory actions of spinal orphanin FQ. Canadian Journal of Physiological Pharmacology, 76, 314-324.

Jiang, Q., Takemori, A.E., Sultana, M., Portoghese, P.S., Bowen, W.D., Mosberg, H.I., & Porreca, F. (1991) Differential antagonism of opioid delta antinociception by [D-Ala2, Leu5, Cys6]-enkephalin (DALCE) and naltrindole 5'-isothiocyanate (5'-NTII): evidence for delta receptor subtypes. Journal of Pharmacology and Experimental Therapeutics, 257, 1069-1075.

Kalivas, P.W., Gau, B.A., Nemeroff, C.B., & Prange, A.J. (1982) Antinociception after microinjection of neurotensin into the central amygdaloid nucleus of the rat. Brain Research, 243, 279-286.

Kastin, A.J., Galina, Z.H., Horvath, A., & Olson, R.D. (1987) Some principles in the peptide field. Journal of Allergy Clinical Immunology, 79, 6-11.

Kavaliers, M., & Perrot-Sinalts, M. (1996) Pronociceptive effects of the neuropeptide, nociceptin, in the land snail. Peptides, 17, 763-768.

Kellstein, D.E., Malseed, R.T., & Goldstein. F.J. (1988) Opioid-monoamine interactions in spinal antinociception: Evidence for serotonin but not norepinephrine reciprocity. Pain, 34, 85-92.

Kenakin, T. (1997) Differnces between natural and recombinant G protien-coupled receptor systems with varying receptor/G protein stoichiometry. Trends in Pharmacological Sciences, 18, 456-464.

Kest, B., Orlowski, M., & Bodnar, R.J. (1992) Endopeptidase 24.15 inhibition and opioid antinociception. Psychopharmacology, 106, 408-416.

Kiefel, J.M., Cooper, M.L., & Bodnar, R.J. (1992a) Inhibition of mesencephalic morphine analgesia by methysergide in the medial ventral medulla of rats. Physiology and Behavior, 51, 201-205.

Kiefel, J.M., Cooper, M.L., & Bodnar, R.J. (1992b) Serotonin receptor subtype antagonists in the medial ventral medulla inhibit mesencephalic opiate analgesia. Brain Research, 597, 331-338.

Kiefel, J.M., Rossi, G.C., & Bodnar, R.J. (1993) Medullary mu and delta opioid receptors modulate mesencephalic morphine analgesia in rats. Brain Research, 624, 151-160.

Kieffer, B.L. (1995) Recent advances in molecular recognition and signal transduction of active peptides: Receptors for opioid peptides. Cellular and Molecular Neurobiology, 15, 615-635.

Kieffer, B.L. (1997) Molecular aspects of opioid receptors. Dickenson, A., & Besson, J.M. Pain. Handbook of Experimental Pharmacology. Springer-Verlag:Berlin, Heidelberg. 281-303.

Kieffer, B., Befort, K., Gareiaux-Ruff, C., & Hirth, C. (1992) The delta-opioid receptor: isolation of cDNA by expression cloning and pharmacological characterization. Proceedings of the National Academy of Science (USA), 89, 12048-12052.

Kimura, S., Lewis, R.V., Stern, A.S., Rossier, J., Stein, S., & Undenfriend, S. (1980) Probable precursors of (leu) and (met)- enkephalin in adrenal medulla: Peptides of 3-5 kilodaltons. Proceedings of the National Academy of Science (USA), 77, 1681-85.

King, M.A., Rossi, G.C., Chang, A.H., Williams, L., & Pasternak, G.W. (1997) Spinal analgesic activity of orphanin FQ/nociceptin and its fragments. Neuroscience Letters, 223, 113-116.

Klamt, J.G., & Prado, W.A. (1991) Antinociception and behavioral changes induced by carbachol microinjected into identified sites of the rat brain. Brain Research, 549, 9-18.

Kolesnikov, Y.A., & Pasternak, G.W. (1999) Peripheral orphanin FQ/nociceptin analgesia in the mouse. Life Sciences, 64, 2021-2028.

Krettak, J.E., & Price, J.L. (1978) Amygdaloid projections to subcortical structures within the basal forebrain and brainstem in the rat and cat. Journal of Comparative Neurology, 178, 225-254.

Kromor, W. (1988) Endogenous and exogonous opioids in the control of gastrointestinal motility and secretion. Pharmacological Reviews, 40, 121-181.

Lasagna, L., & Beecher, H.K. (1954) Analgesic effectiveness of nalorphine and nalorphine-morphine combinations in man. Journal of Pharmacology and Experimental Therapeutics, 112, 356-363.

Letchworth, S.R., Mathis, J.P., Rossi, G.C., Bodnar, R.J., & Pasternak, G.W. (2000) Autoradiographic localization of 125-I[Tyr14]Orphanin FQ/Nociceptin and 125-I[Tyr10]Orphanin FQ/Nociceptin1-11 binding sites in rat brain. Journal of Comparative Neurology, 423, 319-329.

Leventhal, L., Cole, J.L., & Bodnar, R.J. (1996) Reductions in locomotor activity following central opioid receptor subtype antagonists in rats. Physiology and Behavior, 60, 833-836.

Leventhal, L., Mathis, J.P., Rossi, G.C., Pasternak, G.W., & Bodnar, R.J. (1998) Orphan opioid receptor antisense probes block orphanin FQ-induced hyperphagia. European Journal of Pharmacology, 349, R1-R3.

Levine, A.S., Grace, M., Billington, C.J., & Portoghese, P.S. (1990) Nor-binaltorphamine decreases deprivation and opioid-induced feeding. Brain Research, 534, 60-64.

Light, A.R., Trevino, D.L., & Perl, E.R. (1979) Morphological features of functionally identified neurons in the marginal zone and substantia gelatinosa of the spinal dorsal horn. Journal of Comparative Neurology, 186, 151-171.

Loomis, C.W., Jhamandas, K., Milne, B., & Cervenko, F. (1987) Monoamine and opioid interactions in spinal analgesia and tolerance. Pharmacology Biochemistry and Behavior, 26, 445-451.

Lord, J.A.H., Waterfield, A.A., Hughes, J., & Kosterlitz, H. (1977) Endogenous opioid peptides: multiple agonists and receptors. Nature, 267, 495-499.

Ma, Q.-P., Shi, Y.-S., & Han, J.-S. (1992) Further studies on interactions between periaqueductal gray, nucleus accumbens and habenula in antinociception. Brain Research, 583, 292-295.

Mains, R.E., Eipper, B.A., & Ling, N. (1977) Common precursor to corticotropins and endorphin. Proceedings of the National Academy of Science (USA), 1974, 3014-3018.

Mamiya, T., Noda, Y., Nishi, M., Takeshima, H., & Nabeshima, T. (1998) Enhancement of spatial attention in nociceptin/orphanin FQ receptor-knockout mice. Brain Research, 783, 236-240.

Manabe, T., Noda, Y., & Mamiya, T. (1998) Facilitation of lont-term potentiation and memory in mice lacking nociceptin receptors. Nature, 394, 577-581.

Manning, B.H., & Mayer, D.J. (1995a) The central nucleus of the amygdala contributes to the production of morphine antinociception in the tail-flick test. The Journal of Neuroscience, 15, 8199-8213.

Manning, B.H., & Mayer, D.J. (1995b) The central nucleus of the amygdala contributes to the production of morphine antinociception in the formalin test. Pain, 63, 141-152.

Mansour, A., Burke, S., Pavlic, R.J., Akil, H., & Watson, S.J. (1996) Immunohistochemical localization of the cloned kappa-1 receptor in the rat CNS. Neuroscience, 71, 671-690.

Mansour, A., Fox, C.A., Akil, H., & Watson, S.J. (1995) Opioid-receptor mRNA expression in the rat CNS: anatomical and functional implications. Trends in Neuroscience, 18, 22-29.

Mansour, A., Fox, C.A., Burke, S., Akil, H., & Watson, S.J. (1995) Immunohistochemical localization of the cloned mu opioid receptor in the rat CNS. Journal of Chemical Neuroanatomy, 8, 283-305.

Mansour, A., Fox, C.A., Burke, S., Meng, F., Thompson, R.C., Akil, H., & Watson, S.J. (1994) Mu, delta and kappa opioid receptor mRNA expression in the rat CNS: an in situ hybridization study. Journal of Comparative Neurology, 350, 412-438.

Mansour, A., Fox, C.A., Burke, S., & Watson, S.J. (1994) Immunohistochemical localization of the mu opioid receptors. Regulatory Peptides, 54, 179-180.

Mansour, A., Fox, C.A., Thompson, R.C., Akil, H., & Watson, S.J. (1994) Mu-opioid receptor mRNA expression in the rat CNS: comparison to mu-receptor binding. Brain Research, 643, 245-265.

Mansour, A., Khachaturian, H., Lewis, M.E., Akil, H., & Watson, S.J. (1988) Anatomy of CNS opioid receptors. Trends in Neuroscience, 11, 308-314.

Mansour, A., Khachaturian, H., Lewis, M.E., Akil, H., & Watson, S.T. (1987) Autoradiographic differentiation of mu, delta and kappa opioid receptors in the rat forebrain and midbrain. The Journal of Neuroscience, 7, 2445-64.

Marcilhac, A., & Siaud, P. (1996) Indentification of projections from the central nucleus of the amygdala to the paraventricular nucleus of the hypothalamus which are immunoreactive for corticotrophin releasing hormone in the rat. experimental physiology, 82, 273-281.

Martin, W.R., Eades, C.G., Thompson, J.A., Huppler, R.E., & Gilbert, P.E. (1976) The effects of morphine- and nalorphine-like drugs in the nondependent and morphine-dependent chronic spinal dog. Journal of Pharmacology and Experimental Therapeutics, 197, 517-532.

Mattia, A., Farmer, S.C., Takemori, A.E., Sultana, M., Portoghese, P.S., Mosberg, H.I., Bowen, W.D., & Porreca, F. (1992) Spinal opioid delta antinociception in the mouse: mediation by a 5'-NTII-sensitive delta receptor subtype. Journal of Pharmacology and Experimental Therapeutics, 260, 518-525.

Mattia, A., Vanderah, T., Mosberg, H.I., & Porreca, F. (1991) Lack of antinociceptive cross-tolerance between [D-Pen2, D-Pen5]-enkephalin and [D-Ala2]-deltorphan II in mice: evidence for delta receptor subtypes. Journal of Pharmacology and Experimental Therapeutics, 258, 583-587.

McGaugh, J.L., Introini-Collison, I.B., & Castellano, C. (1993) Involvement of opioid peptides in learning and memory. . Handbool of Experimental Pharmacology, Opioids II. Springer-Verlag:Berlin. 429-441.

Meunier, J.C. (1997) Nociceptin/orphanin FQ and the opioid receptor-like ORL-1 receptor. European Journal of Pharmacology, 340, 1-15.

Meunier, J.C. (2000) The potential therapeutic value of nociceptin receptor agonists and antagonists. Expert Opinion in Therapeutics and Patents, 10, 371-388.

Meunier, J.C., Mollereau, C., Toll, L., Suadeau, C., Moisand, C., Alvinerie, P., Butour, J.L., Guillemot, J.C., Ferrara, P., Monsarrat, B., Mazargull, H., Vassart, G., Parmentier, M., & Constentin, J. (1995) Isolation and structure of the endogenous agonist of the opioid receptor like ORL1 receptor. Nature, 377, 532-535.

Millan, M.J. (1990) Kappa-opioid receptors and analgesia. Trends in Pharmacological Sciences, 11, 70-76.

Minami, T., Okuda-Ashitaka, E., Nishizawa, M., Mori, H., & Ito, S. (1997) Inhibition of nociceptin-induced allodynia in conscious mice by prostaglandin D2. British Journal of Pharmacology, 122, 605-610.

Mogil, J.S., Grisel, J.E., Reinscheid, K.K., Civelli, O., Belknap, J.K., & Grandy, D.K. (1996) Orphanin FQ is a functional anti-opioid peptide. Neuroscience, 75, 333-337.

Mogil, J.S., Grisel, J.E., Zhangs, G., Belknap, J.K., & Grandy, D.K. (1996) Functional antagonism of mu, delta and kappa-opioid antinociception by orphanin FQ. Neuroscience Letters, 214, 131-134.

Mollereau, C., Parmentier, M., Mailleux, P., Butour, J.L., Moisand, C., Chalon, P., Caput, D., Vassart, G., & Meunnier, J.C. (1994) ORL-1, a novel member of the opioid family: cloning, functional expression and localization. FEBS Letters, 341, 33-38.

Mollereau, C., Simons, M.J., & Soularue, P. (1996) Structure, tissue distribution, and chromosomal localization of the prepronociceptin gene. Proceedings of the National Academy of Science (USA), 93, 8666-8670.

Monteillet-Agius, G. (1999) Corrigendum. Journal of Comparative Neurology, 412, 708.

Mosberg, H.I., Hurst, R., Hruby, V.J., Gee, K., Yamamura, H.I., Galligan, J.J., & Burks, T.F. (1983) Bis-penicillamine enkephalins possess highly improved specificity towards delta opioid receptors. Proceedings of the National Academy of Science (USA), 80, 5871-5874.

Moskowitz, A.S., & Goodman, R.R. (1985) Autoradiographic analysis of mu-1 and mu-2 and delta opioid binding in the central nervous system of C57BL6BY and CXBK (opioid receptor deficient) mice. Brain Research, 360, 108-116.

Murphy, N.P., Lee, Y., & Maidment, N.T. (1999) Orphanin FQ/nociceptin blocks acquisition of morphine place preference. Brain Research, 832, 168-170.

Neal, C.R., Mansour, A., Reinscheid, R.K., Nothacker, H.P., Civelli, O., & Watson, S.J. (1999) Localization of orphanin FQ (nociceptin) peptide and messenger RNA in the cental nervous system of the rat. Journal of Comparative Neurology, 406, 503-547.

Negri, L., Potenza, R.L., Corsi, R., & Melchiorri, P. (1991) Evidence for two subtypes of delta receptors in rat brain. European Journal of Pharmacology, 196, 335-336.

North, R.A. (1986) Opioid receptor types and membrane ion channels. Trends in Neuroscience, 9, 114-117.

Nothacker, H.P., Reinscheid, R.K., & Mansour, A. (1996) Primary structure and tissue distribution of the orphanin FQ precursor. Proceedings of the National Academy of Science (USA), 93, 8677-8682.

Okuda-Ashitaka, E., Tachibana, S., & Houtani, T. (1996) Identification and characterization of an endogenous ligand for opioid receptor homologue ROR-C: its involvement in allodynic response to innocuous stimulus. Brain Research Molecular Brain Research, 43, 96-104.

Paden, C.M., Krall, S., & Lynch, W.C. (1987) Heterogeneous distribution and upregulation of mu, delta and kappa opioid receptors in the amygdala. Brain Research, 418, 349-355.

Pan, Y.X., Cheng, J., Xu, J., Rossi, G., Jacobson, E., Ryan-Moro, J., Brooks, A.I., Dean, G.E., Standifer, K.M., & Pasternak, G.W. (1995) Cloning and functional characterization through antisense mapping of a kappa3-related opioid receptor. Molecular Pharmacology, 47, 1180-1188.

Pan, Y.X., Xu, J., Mahurter, L., Bolan, E., Xu, M., & Pasternak, G.W. (2001) Generation of the mu opioid receptor (MOR-1) protein by three new splice variants of the Oprm gene. Proceedings of the National Academy of Science (USA), 98, 14084-14089.

Pan, Y.X., Xu, J., & Pasternak, G.W. (1996) Cloning and expression of a cDNA encoding a mouse brain orphanin FQ/nociceptin precursor. Biochemical Journal, 315, 11-13.

Pasternak, G.W. (1993) Pharmacological mechanisms of opioid analgesics. Clinical Neuropharmacology, 16, 1-18.

Pasternak, G.W., & Standifer, K.M. (1995) Mapping of opioid receptors using antisense oligodeoxynucleotides: Correlating their molecular biology and pharmacology. Trends in Pharmacological Sciences, 16, 344-350.

Pasternak, G.W., & Wood, P.L. (1986) Multiple mu opiate receptors. Life Sciences, 38, 1889-1896.

Paul, D., Bodnar, R.J., Gistrak, M.A., & Pasternak, G.W. (1989) Different mu receptor subtypes mediate spinal and supraspinal analgesia in mice. European Journal of Pharmacology, 168, 307-314.

Paul, D., Pick, C.G., Tive, L.A., & Pasternak, G.W. (1991) Pharmacological characterization of nalorphine, a k3 analgesic. Journal of Pharmacology and Experimental Therapeutics, 257, 1-7.

Pavlovic, Z.W., & Bodnar, R.J. (1998a) Opioid supraspinal analgesic synergy between the amygdala and periaqueductal gray in rats. Brain Research, 779, 158-169.

Pavlovic, Z.W., & Bodnar, R.J. (1998b) U50488H-induced analgesia in the amygdala: test-specific effects and blockade by opioid antagonists in the periaqueductal gray. Analgesia, 3, 223-230.

Pavlovic, Z.W., Cooper, M.L., & Bodnar, R.J. (1996a) Enhancements in swim stress-induced hypothermia, but not analgesia following amygdala lesions in rats. Physiology and Behavior, 59, 77-82.

Pavlovic, Z., Cooper, M.L., & Bodnar, R.J. (1996b) Opioid antagonists in the periaqueductal gray inhibit morphine and beta-endorphin analgesia elicited from the amygdala of rats. Brain Research, 741, 13-26.

Pazos, A., & Palacios, J.M. (1985) Quantitative autoradiographic mapping of serotonin receptors in the rat brain. I. Serotonin-1 receptors. Brain Research, 346, 205-230.

Pert, C.B., & Snyder, S.H. (1973) Opiate receptor: demonstration in nervous tissue. Science, 179, 1011-1014.

Pickel, V.M., & Colago, E.E. (1999) Presence of mu-opioid receptors in targets of efferent projections from the central nucleus of the amygdala to the nucleus of the solitary tract. Synapse, 33, 141-152.

Pomonis, J.D., Billington, C.J., & Levine, A.S. (1996) Orphanin FQ, agonist of orphan opioid receptor ORL1, stimulates feeding in rats. Neuroreport, 8, 369-371.

Porreca, F., Heyman, J.S., Mosberg, H.I., Omnaas, J.R., & Vaught, J.L. (1987) Role of mu and delta receptors in the supraspinal and spinal analgesic effects of (D-Pen2, D-Pen5)-enkephalin in the mouse. Journal of Pharmacology and Experimental Therapeutics, 241, 393-400.

Porreca, F., Mosberg, H.I., Hurst, R., Hruby, V.J., & Burks, T.F. (1984) Roles of mu, delta and kappa opioid receptors in spinal and supraspinal mediation of gastrointestinal

transit effects and hot-plate analgesia in the mouse. Journal of Pharmacology and Experimental Therapeutics, 230, 341-348.

Portoghese, P.S., Larson, D.L., Sayre, L.M., Fries, D.S., & Takemori, A.E. (1980) A novel opioid receptor site directed alkylating agent with irreversible narcotic antagonistic and reversible agonistic activities. Journal of Medicinal Chemistry, 23, 233-234.

Portoghese, P.S., Lipkowski, A.W., & Takemori, A.E. (1987) Binaltorphamine and nor-binaltorphamine, potent and selective K-opioid receptor antagonists. Life Sciences, 40, 1287-1292.

Portoghese, P.S., Sultana, M., Nagase, H., & Takemori, A.E. (1992) A highly selective d1 opioid receptor antagonist: 7-benzylidenenaltrexone (BNTX). European Journal of Pharmacology, 218, 195-196.

Prieto, G.J., Cannon, J.T., & Liebeskind, J.C. (1983) Nucleus raphe magnus lesions disrupt stimulation produced analgesia from ventral but not dorsal midbrain areas in the rat. Brain Research, 261, 53-57.

Probst, W.C., Snyder, L.A., Schuster, D.I., Brosius, J., & Sealfon, S.C. (1992) Sequence alignment of the G-protein-coupled receptor superfamily. DNA Cell Biology, 11, 1-20.

Rapaka, R.S., & Porreca, F. (1991) Development of delta opioid peptides as nonaddicting analgesics. Pharmacological Research, 8, 1-8.

Reddy, S.V.R., Maderdrut, J.L., & Yaksh, T.L. (1980) Spinal cord pharmacology of adrenergic agonist-mediated antinociception. Journal of Pharmacology and Experimental Therapeutics, 213, 525-533.

Reddy, S.V.R., & Yaksh, T.L. (1980) Spinal noradrenergic terminal system mediates antinociception. Brain Research, 189, 391-404.

Rees, D.C., & Hunter, J.C. (1990) Opioid receptors. . Comprehensive medicinal chemistry. pergamon press:new york. 805-846.

Reinscheid, R.K., Nothacker, H.P., Bourson, A., Ardati, A., Henningsen, R.A., Bunzow, J.R., Grandy, D.K., Langen, H., Monsma, F.J., & Civilli, O. (1995) Orphanin FQ: a neuropeptide that activates an opioid-like G protein-coupled receptor. Science, 270, 792-794.

Reisine, T. (1995) opiate receptors. Neuropharmacology, 34, 463-472.

Reisine, T., & Bell, G.I. (1993) Molecular biology of opioid receptors. Trends in Neuroscience, 16, 506-510.

Riedl, M., Shuster, S., Vulchanova, L., Wang, J., Loh, H.H., & Elde, R. (1996) Orphanin FQ/nociceptin-immunoreactive nerve fibers parallel those containing endogenous opioids in rat spinal cord. Neuroreport, 7, 1369-1372.

Rizvy, T.A., Ennis, M., Behbehani, M.M., & Shipley, M.T. (1991) Connections between the central nucleus of the amygdala and the midbrain periaqueductal gray: topography and reciprocity. Journal of Comparative Neurology, 303, 121-131.

Rochford, J., & Dawes, P. (1993) Effect of naloxone on the habituation of novelty-induced hypoalgesia: the collateral inhibition hypothesis revisitied. Pharmacology Biochemistry and Behavior, 46, 117-123.

Rodgers, R.J. (1977) Elevation of aversive threshold in rats by intra-amygdaloid injection of morphine sulphate. Pharmacology Biochemistry and Behavior, 6, 385-390.

Rodgers, R.J. (1978) Influence of intra-amygdaloid opiate injections on shock thresholds, tail-flick latencies and open field behaviour in rats. Brain Research, 153, 211-216.

Rossi, G.C., Leventhal, L., Bolan, E., & Pasternak, G.W. (1997) Pharmacological characterization of orphanin FQ/nociceptin and its fragments. Journal of Pharmacology and Experimental Therapeutics, 282, 858-865.

Rossi, G.C., Leventhal, L., & Pasternak, G.W. (1996) Naloxone-sensitive orphanin FQ-induced analgesia in mice. European Journal of Pharmacology, 311, R7-R8.

Rossi, G.C., Pan, Y.X., Brown, G.P., & Pasternak, G.W. (1995) Antisense mapping the MOR-1 receptor: evidence for alternative splicing and a novel morphine-6Beta-glucuronide receptor. FEBS Letters, 369, 192-196.

Rossi, G.C., Pasternak, G.W., & Bodnar, R.J. (1993) Synergistic brainstem interactions for morphine analgesia. Brain Research, 624, 171-180.

Rossi, G.C., Pasternak, G.W., & Bodnar, R.J. (1994) Mu and delta opioid synergy between the periaqueductal gray and the rostro-ventral medulla. Brain Research, 665, 85-93.

Rossi, G.C., Pellegrino, M., Shane, R., Abbadie, C.A., Dustman, J., Jimenez, C., Bodnar, R.J., Pasternak, G.W., & Allen, R.G. (2002) Characterization of rat prepro-orphanin FQ/nociceptin(154-181): Nociceptive processing in supraspinal sites. Journal of Pharmacology and Experimental Therapeutics, 300, 257-264.

Rossi, G.C., Pellegrino, M., Shane, R., Abbadie, C.A., Dustman, J., Jimenez, C., Bodnar, R.J., Pasternak, G.W., & Allen, R.G. (2002) Characterization of rat prepro-orphanin FQ/nociceptin(154-181): Nociceptive processing in supraspinal sites. Journal of Pharmacology and Experimental Therapeutics, 300, 257-264.

Rossi, G.C., Perlmutter, M., Leventhal, L., Talatti, A., & Pasternak, G.W. (1998) Orphainin FQ/nociceptin analgesia in the rat. Brain Research, 792, 327-330.

Rossier, J. (1993) Biosynthesis of enkephalins and proenkephalin-derived peptides. . Handbook of Experimental Pharmacology, Opioids I. Springer-Verlag:Berlin. 423-441.

Sandin, J., Georgieva, J., Schott, P.A., Ogren, S.O., & Terenius, L. (1997) Nociceptin/orphanin FQ microinjected into hippocampus impairs spaial learning in rats. European Journal of Neuroscience, 9, 194-197.

Sandkuhler, J., & Gebhart, G.F. (1984) Relative contributions of the nucleus raphe magnus and adjacent medullary reticular formation to the inhibition by stimulation in the periaqueductal gray of a spinal nociceptive reflex in the pentobarbital-anesthetized rat. Brain Research, 305, 77-87.

Satoh, M., Kubota, A., Iwama, T., Wada, T., Yasui, M., Fujibayashi, K., & Takagi, H. (1983) Comparison of analgesic potencies of mu, delta and kappa agonists locally applied to various CNS regions relevant to analgesia in rats. Life Sciences, 33, 689-692.

Schmauss, C., & Yaksh, T.L. (1984) In vivo studies on spinal opiate receptor systems mediating antinociception. II. Pharmacological profiles suggesting a differential association of mu, delta and kappa receptors with visceral chemical and cutaneous thermal stimuli in the rat. Journal of Pharmacology and Experimental Therapeutics, 228, 1-12.

Schulz, S., Schreff, M., Nuss, D., Gramsch, C., & Hollt, V. (1996) Nociceptin/orphanin FQ and opioid peptides show overlapping distribution but not co-localization in pain-modulatory brain regions. Neuroreport, 7, 3021-3025.

Shane, R., Lazar, D.A., Rossi, G.C., Pasternak, G.W., & Bodnar, R.J. (2001) Analgesia elicited by OFQ/nociceptin and its fragments from the amygdala in rats. Brain Research, 907(1-2), 109-116.

Shane, R., Rossi, G.C., Allen, R., Mathis, J.P., Pasternak, G.W., & Bodnar, R.J. (1999) Analgesic actions of orphanin FQ/nociceptin fragments in the ventrolateral periaqueductal gray and amygdala in rats. Society for Neuroscience Abstracts, 25, 1438.

Shane, R., Wilk, S., & Bodnar, R.J. (1999) Modulation of endomorphin-2-induced analgesia by dipeptidyl peptidase IV. Brain Research, 815, 278-286.

Shipley, M.T., Ennis, M., Rivzi, T.A., & Behbehani, M. (1991) Topographical specificity of forebrain inputs to the midbrain periaqueductal gray: evidence for discrete longitudinally organized input columns. Depaulis, A., & Bandler, R. The midbrain periaqueductal gray matter: Functional, anatomical and neurochemical organization. Plenum:New York. .

Shook, J.E., Watkins, W.D., & Camporesi, E.M. (1990) Differential roles of opioid receptros in respiration, respiratory disease, and opiate-induced respiratory depression. American Review of Respiratory Disease, 142, 895-909.

Silverman, A.J., Hoffman, D.L., & Zimmerman, E.A. (1981) The descending afferent connection of the paraventricular nucleus of the hypothalamus (PVN). Brain Research Bulletin, 6, 47-61.

Sim, L.J., & Childers, S.R. (1997) Anatomical distribution of mu, delta and kappa opioid and nociceptin/orphanin FQ stimulated [35S] guanylyl-5'-O-(gamma-Thio)-triphosphate binding in guinea pig brain. Journal of Comparative Neurology, 386, 562-572.

Simon, E.J., Hiller, J.M., & Edelman, I. (1973) Stereospecific binding of the potent narcotic analgesic (3H)etorphine to rat brain homogenate. Proceedings of the National Academy of Science (USA), 70, 1947-49.

Simonin, F., Gavleriaux-Ruff, C., Befort, K., Matthes, H., Lannes, B., Micheletti, G., Mattei, M.G., Charron, G., Bloch, B., & Kieffer, B. (1995) Kappa opioid recptor in humans: cDNA and genomic cloning, chromosomal assignment, functional expression, pharmacology and expression pattern in the central nervous system. Proceedings of the National Academy of Science (USA), 92, 7006-7010.

Smith, D.J., Perotti, J.M., Crisp, T., Cabral, M.E.Y., Long, J.T., & Scalziti, J.N. (1988) The mu receptor is responsible for descending pain inhibition originating in the periaqueductal gray region of the rat brain. European Journal of Pharmacology, 156, 47-54.

Solomon, R.E., & Gebhart, G.F. (1987) Intrathecal clonidine and morphine in rats: Antinociceptive tolerance and cross-tolerance and effects on blood pressure. Pain, Supplements, 4, S41.

Spinella, M., Cooper, M.L., & Bodnar, R.J. (1996) Excitatory amino acid antagonists in the rostral ventromedial medulla inhibit mesencephalic morphine analgesia in rats. Pain, 64, 545-552.

Spinella, M., Schaefer, L.A., & Bodnar, R.J. (1997) Ventral medullary mediation of mesencephalic morphine analgesia by muscarinic and nicotinic cholinergic receptor antagonists in rats. Analgesia, 3, 119-130.

Spinella, M., Znamensky, V., Moroz, M., Ragnauth, A., & Bodnar, R.J. (1999) Actions of NMDA and cholinergic receptor antagonists in the rostral ventromedial medulla upon beta-endorphin analgesia elicited from the ventrolateral periaqueductal gray. Brain Research, 829, 151-159.

Spreekmeester, E., & Rochford, J. (2000) selective mu and delta, but not kappa, opiate receptor antagonists inhibit the habituation of novelty-induced hypoalgesia in the rat. Psychopharmacology, 148, 99-105.

Strader, C.D., Fong, T.M., Tota, M.R., & Underwood, D. (1994) Structure and function of G protein-coupled receptors. Annual Review of Biochemistry, 63, 101-132.

Stratford, T.R., Holahan, M.R., & Kelley, A.E. (1997) Injections of nociceptin into nucleus accumbens shell or ventromedial hypothalamic nucleus increase food intake. Neuroreport, 8, 423-426.

Sutters, K.A., Miaskowski, C., Taiwo, Y.O., & Levine, J.D. (1990) Analgesic synergy and improved motor function produced by combinations of mu-delta and mu-kappa opioids. Brain Research, , 290-294.

Takemori, A.E., Larson, D.L., & Portoghese, P.S. (1981) The irreversible narcotic antagonist and reversible agonistic properties of the fumarate methyl ester derivative of naltrexone. European Journal of Pharmacology, 70, 445-451.

Terenius, L. (1973) Stereospecific interaction between narcotic analgesia and a synaptic plasma membrane fraction of rat cerebral cortex. acta pharmacologica et toxicilogica, 32, 317-320.

Tershner, S.A., & Helmstetter, F.J. (1995) Spinal antinociception following stimulation of the amygdala depends on opioid receptors in the ventral periaqueductal gray. Society for Neuroscience Abstracts, 21, 1169.

Tershner, S.A., & Helmstetter, F.J. (2000) Antinociception produced by mu opioid receptor activation in the amygdala is partly dependent on activation of mu opioid and neurotensin receptors in the ventral periaqueductal gray. Brain Research, 865, 17-26.

Tian, J.H., Xu, W., Fang, Y., Mogil, J.S., Grisel, J.E., Grandy, D.K., & Han, J.S. (1997) Bidirectional modulatory effect of orphanin FQ on morphine-induced analgesia: antagonism in brain and potentiation in spinal cord of the rat. British Journal of Pharmacology, 120, 676-680.

Traynor, J. (1989) Subtypes of the k-opioid receptor: Fact of fiction? Trends in Pharmacological Sciences, 10, 52-53.

Traynor, J.R., & Elliot, J. (1993) Delta-opioid receptor subtypes cross talk with mu-receptors. Trends in Pharmacological Sciences, 14, 84-85.

Tribollet, E., & Dreifuss, J.J. (1981) Localization of neurones projecting to the hypothalamic paraventricular nucleus of the rat: a horseradish peroxidase study. Neuroscience, 7, 1215-1328.

Tseng, L.F. (2001) Evidence for epsilon-opioid receptor mediated beta-endorphin-induced analgesia. Trends in Pharmacological Sciences, 22(12), 623-630.

Tung, A.S., Yaksh, T.L., & Wang, J.Y. (1981) Tolerance to intrathecal opiates in the rat. Anesthesiology, 55, A171.

Ueda, H., Yamaguchi, T., Tokuyama, S., Inoue, M., Nishi, M., & Takeshima, H. (1997) Partial loss of tolerance liability to morphine tolerance in mice lacking the nociceptin receptor gene. Neuroscience Letters, 237, 136-138.

Uhl, G.R., Childers, S.R., & Pasternak, G.W. (1994) An opiate receptor gene family reunion. Trends in Neuroscience, 17, 89-93.

Urban, M.O., & Smith, D.J. (1993a) Role of neurotensin in the nucleus raphe magnus in opioid-induced antinociception from the periaqueductal gray. Journal of Pharmacology and Experimental Therapeutics, 265, 580-586.

Urban, M.O., & Smith, D.J. (1993b) RVM nuclei that mediate morphine antinociception from the PAG. Society for Neuroscience Abstracts, 19, 1405.

Urban, M.O., & Smith, D.J. (1994a) Localization of the antinociceptive and antianalgesic effects of neurotensin within the rostral ventromedial medulla. Neuroscience Letters, 174, 21-25.

Urban, M.O., & Smith, D.J. (1994b) Nuclei within the rostral ventromedial medulla mediating morphine antinociception from the periaqueductal gray. Brain Research, 652, 9-16.

Vanderah, T., Takemori, A.E., Sultana, M., Portoghese, P.S., Mosberg, H.I., Hruby, V.J., Haaseth, R.C., Matsunaga, T.O., & Porreca, F. (1994) Interaction of [D-Pen2, D-Pen5]-enkephalin and [D-Ala2, Glu4]-deltorphin with delta-opioid receptor subtypes in vivo. European Journal of Pharmacology, 252, 133-137.

vanPraag, H., & Frenk, H. (1990) The role of glutamate in opiate descending inhibition of nociceptive spinal reflexes. Brain Research, 524, 101-105.

VanVoigtlander, P.F., Lahti, R.A., & Ludens, J.H. (1983) U50488H: a selective and structurally novel non-mu (kappa) opioid agonist. Journal of Pharmacology and Experimental Therapeutics, 224, 7-11.

Vaupel, B. (1983) Naloxone fails to antagonize the delta effects of PCP and SKF 10,047 in the dog. European Journal of Pharmacology, 92, 269-274.

Waeber, C., Dixon, K., Hoyer, D., & Palacios, J.M. (1988) Localization by autoradiography of 5HT-3 receptors in the mouse CNS. European Journal of Pharmacology, 151, 351-352.

Walker, J.R., & Koob, G.F. (1997) Orphan anxiety. Proceedings of the National Academy of Science (USA), 94, 14217-14219.

Walker, J.R., Spina, M., Terenius, L., & Koob, G.F. (1998) Nociceptin fails to affect heroin self-administration in the rat. Neuroreport, 9, 2243-2247.

Wang, J.B., Johnson, P.S., Imai, Y., Persico, A.M., Ozenberger, B.A., Eppler, C.M., & Uhl, G.R. (1994) cDNA cloning of an orphan opiate receptor gene family member and its splice variant. FEBS Letters, 348, 75-79.

Wang, J.K. (1977) Antinociceptive effect of intrathecally administered serotonin. Anesthesiology, 47, 269-271.

Watkins, L.R., Wiertelak, E.P., & Maier, S.F. (1993) The amygdala is necessary for the expression of conditioned but not unconditioned analgesia. Behavioral Neuroscience, 107, 402-405.

Weller, K.L., & Smith, D.A. (1982) Afferent connections to the bed nucleus of the stria terminalis. Brain Research, 232, 255-270.

Wick, M.J., Minnerath, S.R., Lin, X., Elde, R., Law, P.-Y., & Loh, H.H. (1994) Isolation of a novel cDNA encoding a putative membrane receptor with high homology to the cloned, mu, delta and kappa opioid receptors. Molecular Brain Research, 27, 37-44.

Wuster, M., Schulz, R., & Herz, A. (1980) Opiate activity and receptor selectivity of dynorphin(1-13) and related peptides. Neuroscience Letters, 20, 79-83.

Xu, X.J., Hao, J.X., & Wiesenfeld-Hallin, Z. (1996) Nociceptin or antinociceptin: potent spinal antinociceptive effect of orphanin FQ/nociceptin in the rat. Neuroreport, 7, 2092-2094.

Yaksh, T.L. (1981) Spinal opiate analgesia: characteristics and principles of action. Pain, 11, 293-296.

Yaksh, T.L. (1984a-a) Multiple opioid receptor systems in the brain and spinal cord. I. European Journal of Anesthesiology, 1, 171-201.

Yaksh, T.L. (1984b-b) Multiple opioid receptor systems in the brain and spinal cord. II. European Journal of Anesthesiology, 1, 201-243.

Yaksh, T.L., & Rudy, T.A. (1978) Narcotic analgetics: CNS sites on mechanisms of action as revealed by intracerebral injection techniques. Pain, 4, 299-359.

Yaksh, T.L., & Wilson, P.R. (1979) Spinal serotonin terminal system mediates antinociception. Journal of Pharmacology and Experimental Therapeutics, 208, 446-453.

Yamamoto, T., Nozaki-Taguchi, N., & Kimura, S. (1997) Effects of intrathecally administered nociceptin, an opioid receptor-like1 (ORL1) receptor agonist, on the thermal hyperalgesia induced by unilaeral constriction injury to the sciatic nerve in the rat. Neuroscience Letters, 224, 107-110.

Yeung, J.C., & Rudy, T.A. (1980a) Multiplicative interaction between narcotic agonisms expressed at spinal and supraspinal sites of antinociceptive action as revealed by concurrent intrathecal and intracerebroventricular injections of morphine. Journal of Pharmacology and Experimental Therapeutics, 215, 633-642.

Yeung, J.C., & Rudy, T.A. (1980b) Sites of antinociceptive action of systemically-injected morphine: involvement of supraspinal loci as revealed by intracerebroventricular injection of naloxone. Journal of Pharmacology and Experimental Therapeutics, 215, 626-632.

Young, E., Bronstein, D., & Akil, H. (1993) Proopiomelanocortin biosynthesis, processing and secretion: functional implications. . Handbook of Experimental Pharmacology, Opioids I,. Springer-Verlag:Berlin. 393-413.

Yu, T.P., Fein, J., Phan, T., Evans, C.J., & Xie, C.W. (1997) Orphanin FQ inhibits synaptic transmission and long-term potentiation in rat hippocampus. Hippocampus, 7, 88-94.

Zadina, J.E., Hackler, L., Ge, L.-J., & Kastin, A.J. (1997) A potent and selective endogenous agonist for the mu-opiate receptor. Nature, 386, 499-502.

Zukin, R.S., Brady, K.T., Slifer, B.L., & Balster, R.L. (1984) Behavioral and biochemical steroselectivity of sigma opiate/PCP receptors. Brain Research, 294, 174-177.

Zukin, R.S., Eghbalai, M., Olive, D., Unterwald, E.M., & Tempel, A. (1988) Characterization and visualization of rat and guinea pig brain K opioid receptors: evidence for K-1 and K-2 opioid receptors. Proceedings of the National Academy of Science (USA), 85, 4061-4065.

Zukin, R.S., & Zukin, S.R. (1981) Multiple opiate receptors: emerging concepts. Life Sciences, 29, 2681-2690.