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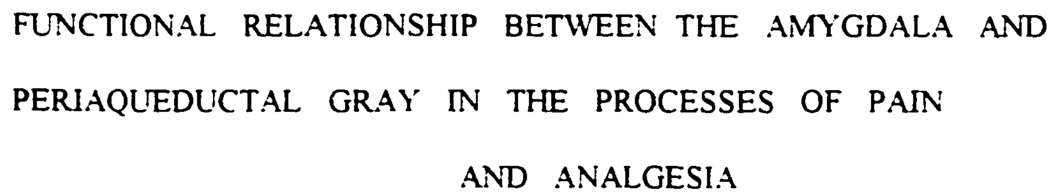

FUNCTIONAL RELATIONSHIP BETWEEN THE AMYGDALA AND PERIAQUEDUCTAL GRAY IN THE PROCESSES OF PAIN AND ANALGESIA

by Zoran Pavlovic

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.

1998

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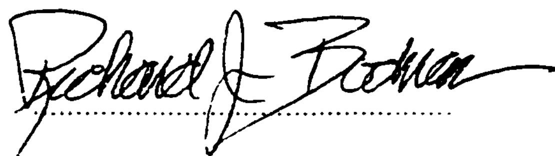

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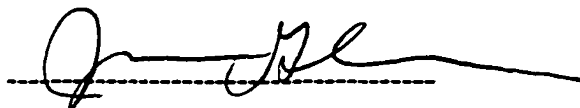

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Abstract

FUNCTIONAL RELATIONSHIP BETWEEN THE AMYGDALA AND PERIAQUEDUCTAL GRAY IN THE PROCESSES OF PAIN AND ANALGESIA

by

Zoran Pavlovic

Mentor: Dr. Richard J. Bodnar

The amygdala is a temporal lobe structure that is involved in the processes of fear and anxiety, conditional emotional responses, learning and memory, aggression, stress, and analgesia It has extensive two-way projections with brainstem structures, such as the periaqueductal gray (PAG), which is part of endogenous antinociceptive circuitry.

Microinjections of either morphine and beta-endorphin in the amygdala significantly increased tail-flick latencies and jump thresholds. The increases were far more pronounced on the jump test than on the tail-flick test. Placements dorsal and medial to the amygdala were ineffective. PAG preatretment with either general (naltrexone), $delta_2$ (NTII) and to a lesser degree mu (BFNA) opioid antagonists significantly reduced both morphine and beta-endorphin analgesia elicited from the amygdala. These data indicate that PAG $delta_2$ and to a lesser degree mu opioid receptors are necessary for the full expression of morphine and beta-endorphin analgesia elicited from the amygdala.

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This dissertation then characterized functional analgesic interactions between the amygdala and the PAG by examining multiplicative interactions for sub-effective doses of morphine and beta-endorphin simultaneously applied to these two structures. Co-administration of subthreshold doses of either morphine or beta-endorphin into both the amygdala and the PAG, as well as when beta-endorphin was microinjected in the amygdala and morphine in the PAG, produced a potent synergistic interaction. However, co-administration of morphine in the amygdala and beta-endorphin in the PAG failed to produce an interactive effect. This result argues for multiple modulatory mechanisms mediating beta-endorphin analgesia in the PAG, which is not the same as for morphine. Co-administration of subeffective doses of morphine and beta-endorphin into either the amygdala alone or the PAG alone failed to produce an interaction, thus indicating the importance of regional opioid activation.

Finally, microinjections of the kappa1 opioid agonist, U58488H in the amygdala elicited test-specific analgesia on the jump test and not the tail-flick test. Like morphine and beta-endorphin analgesia, U50488H-induced analgesia elicited from the amygdala was blocked by PAG preatretment with general and mu, but not delta antagonists, indicating mediation by mu opioid synapses in the PAG.

These data collectively implicate the amygdala as a part of endogenous antinociceptive circuitry and points towards its elaborate functional analgesic interactions with the PAG. These data also integrate opioid analgesic systems in the amygdala with the structure's previously known roles in fear and stress.

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Glossary of Abbreviated Terms

1. PAG= periaqueductal gray
2. DLP= dorsal lateral pons
3. RVM= rostral ventral medulla
4. LC= locus ceruleus
5. DLF= dorsal lateral funiculus
6. NRGC= nucleus reticularis gigantocellularis
7. CEA= central amygdaloid nuclei
8. BLA= baso-lateral amygdaloid nuclei
9. BMA= baso-medial amygdaloid nuclei
10. DPDPE= D-Pen^2,D-Pen^5-enkephalin; delta$_1$ agonist
11. DELT= D-Ala^2,Glu^4-deltorphin; delta$_2$ agonist
12. U50488H= prototypical kappa agonist
13. β-FNA= beta-funaltrexamine, mu antagonist
14. Nor-BNI= nor-binaltorphamine, kappa antagonist
15. DALCE= D-Ala^2, Leu^5,Thr^6-enkephalin, delta antagonist
16. DADLE= [^{3}H] D Ser^2, Leu^5, Thr^6-enkephalin, general delta agonist
17. DSLET= [^{3}H] D-Ala^2, D-Leu^5-enkephalin, general delta agonist
18. ICI174864= general delta antagonist
19. NTII= naltrindole-isothiocianate, delta selective, non-peptide antagonist
20. POMC= pro-opiomelanocortin
21. BNTX= 7-benzylidenenaltrexone, d$_1$antagonist
22. IOXY= heneral opioid antagonist, the naltrexone analogue
23. ACTH= adrenocorticorticotropin
24. AMSH= alpha-melanocyte-stimulating-hormone
25. β-LPH= beta lipotropin
26. Gamma-MSH= Gamma-melanocyte-stimulating hormone
27. EKC= ethylketocyclazocine
28. ng= nanogram
29. ug= microgram
30. i.t.= intrathecal
31. i.c.v.= intracerebroventricular
32. i.c.= intracerebral
33. IP= intraperitoneal
34. IM= intramascular
35. IV= intravenous
36. SIA= stress induced analgesia

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I would like to use this opportunity to thank my mentor, Dr. Richard Bodnar who helped me with his instruction and guidance from the beginning to the end of this dissertation. His enthusiasm and insight into neuroscience are hardly to be matched.

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I. INTRODUCTION

Since the discovery of opioid peptide gene-related families and multiple opioid receptor subtypes in the early 1970's, research has focused on the delineation and characterization of endogenous pain-inhibitory systems that produce centrifugal opioid inhibition of nociceptive input at both the supraspinal and spinal levels of the neuraxis. Numerous studies have implicated mesencephalic, metencephalic and mylencephalic loci involved in the mediation of opioid-induced analgesia, including the periaqueductal gray (PAG), locus coeruleus (LC), nucleus raphe magnus (NRM) and the nucleus reticularis gigantocellularis (NRGC) (see review: Fields and Basbaum,1984). Fields and Basbaum (Fields and Basbaum, 1978; Basbaum and Fields, 1984; Fields at al., 1991) proposed that endogenous opioid pain-inhibition originates in the ventral and ventrolateral mesencephalic PAG and adjacent dorsal raphe nucleus, projects to the rostroventromedial medulla (RVM) which includes the NRM and NRGC, and sends descending projections through the dorsolateral funniculus to the superficial nuclei of the dorsal horn of the spinal cord.

In addition to the brain stem and spinal cord, some forebrain structures such as the arcuate and lateral hypothalamic nuclei and the amygdala have also been implicated in opioid analgesic processes. The amygdala is known to have wide-spread anatomical connections with brain stem structures involved in opioid analgesic processes, most notably with the PAG (Petrov et al., 1995; Grey et al., 1991; Tamara Da Costa Gomez, 1995; Krettek and Price, 1978). The PAG appears to be a critical link for the involvement of the amygdala in opioid-mediated pain-inhibitory processes (Tershner and Helmstetter,

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1995). The results of neurochemical and electrophysiological studies reveal the existence of both excitatory and inhibitory projections from the amygdala to the PAG, and that both pathways involve μ and/or δ-opioid receptors (Tamara Da Costa Gomez, 1995). The purpose of the present dissertation is to characterize opioid receptor subtypes responsible for analgesia in the amygdala, and to investigate further potential functional connections between the amygdala and the PAG. Three series of specific experiments were conducted:

1. The first study determined whether the prototypical μ opioid agonist morphine (Lord at al., 1977; Martin at al., 1976) and the C-terminal fragment of beta-lipotropin, beta-endorphin elicited analgesia following microinjections in the amygdala. Then, the role of opioid receptor subtypes in the PAG were examined for their ability to mediate analgesia elicited by morphine and beta-endorphin in the amygdala. Mu opioid agonists produce potent analgesia in the amygdala on both radiant heat (Helmstetter et al., 1994) and shock (Rodgers, 1977) nociceptive measures. Since different neurochemical systems have been proposed to mediate morphine and beta-endorphin in both supraspinal and spinal structures (see review: Tseng, 1995), the present experiment evaluated this relationship in the amygdala.

2. The second study determined the presence of functional multiplicative analgesic interactions between the amygdala and PAG when subthreshold doses of either morphine were administered simultaneously to each of the sites, beta-endorphin were administered simultaneously to each of the sites, or when subthreshold doses of morphine were administered to one site and beta-endorphin was administered to the second site.

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Such multiplicative interactions have been observed between the PAG and the LC, the PAG and the RVM and the RVM and the LC (Rossi et al., 1993, 1994). Comparisons of interactions between subthreshold doses of morphine and beta-endorphin in the amygdala and PAG provided further insight into their different neurochemical substrates mediating analgesia (see review: Tseng, 1995).

3. The third study determined whether microinjections of the selective κ_1 opioid-receptor agonist U50,488H into the amygdala produced analgesia, and whether an opioid synapse in the PAG mediated this response. Although kappa agonists elicit analgesia on the tail-flick test after ventricular injections (e.g. Van Voigtlander et al., 1983; Cox and Goldstein, 1981), no supraspinal sites, including the amygdala, have been identified to support this response (Bodnar et al., 1991; Rossi et al., 1994; Helmstetter et al., 1995). To provide an underlying conceptual basis, background, and rationale for these experiments, the following sections of the Introduction will examine:

a. the classification of endogenous opioid peptide families,

b. the description of multiple opioid receptor subtypes, including relevant selective agonists and antagonists,

c. the description of an endogenous opioid pain-inhibitory system, including its physiology and pharmacology, its supraspinal and spinal sites of action and its functional interrelationship between different opioid systems along the neuraxis

d. the role of the amygdala in the processes of fear, anxiety, aggression, stress, emotional memory and learning, and pain and analgesia, and

e. a rationale for the present experiments.

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A. CLASSIFICATION OF ENDOGENOUS OPIOID PEPTIDE FAMILIES

The discovery of opioid receptors in the CNS (Pert and Snyder, 1973; Simon et al., 1973) prompted an intense search for their endogenous ligands. The first endogenous opioid peptides to be described were Leu-enkephalin (Leu-Enk) and Met-Enk (Met-Enk) (Hughes et al., 1975), soon followed by characterization of β-endorphin and related peptides (Guillemin et al., 1976), and dynorphin and related peptides (Goldstein et al., 1979). The subsequent identification of the amino acids sequences of the opioid peptides revealed a common Tyr-Gly-Gly-Phe core sequence present in these opioid peptides, and demonstrated that these peptides were derived from three prehormone precursors, namely proopiomelanocortin (POMC), proenkephalin (ProEnk), and prodynorphin (ProDyn) (see review: Akil et al., 1984). Each opioid peptide is first synthesized as a part of a larger inactive prohormone precursor, and is subsequently cleaved at basic residues (e.g. arginyl-arginine, lysyl-lysine, or lysyl-arginine signal specific cleavage) to release a biologically active peptide (Young et al., 1993). The extent of peptide processing, and the resulting peptide products that are ultimately released, depends on the cell in which they are localized, the physiological state of the cell and their recent secretory demand (Mansour et al., 1995).

1.) Proopiomelanocortin (POMC) POMC is the common precursor for beta-endorphin and its precursor beta-lipotropin (beta-LPH), adrenocorticotropic hormone (ACTH) and related bioactive peptides (Mains and Eipen, 1977; Roberts and Herbert, 1978). $ACTH_{1-39}$ is further cleaved into alpha-melanocyte-stimulating hormone (alpha-MSH) and corticotropin-like intermediate lobe peptide (CLIP) (Brownstein, 1980). Beta-

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endorphin, the 31-residue C-terminal component of B-LPH is generated from the post-translational process of B-LPH from proopiomelanocortin (Guillemin et al., 1976). Beta-endorphin $_{1\text{-}31}$ displays a good affinity for μ and δ receptors and a comparatively low affinity for κ receptors (Mansour et al., 1995). Further processing of B-endorphin $_{1\text{-}31}$ to B-endorphin $_{1\text{-}27}$ transforms the peptide to an antagonist with small changes in affinity for μ and δ receptors and no change in receptor selectivity (Nicholas et al., 1985).

In the brain, POMC-derived peptides are located in two distinct cell groups: the arcuate and periarcuate nuclei of the hypothalamus and the caudal part of the nucleus tractus solitarius (Khachaturian et al., 1985). POMC cells in the arcuate nucleus project rostrally through periventricular diencephalic and telencephalic areas, innervating the preoptic area, **amygdala**, septum and the bed nucleus of stria terminalis. Caudally-projecting POMC innervate the periventricular thalamus, PAG, NRM, NRGC, NTS, nuclei reticularis lateralis, parabrachialis and ambiguus, and the dorsal motor nucleus of the vagus (Khachatturian et al., 1985).

2.) Pre-Pro-enkephalin Pre-Pro-enkephalin contains four copies of met-enkephalin, and one of each of leu-enkephalin and the C-terminally extended peptides of Met-enkephalin-Arg-Gly-Leu and Met-enkephalin-Arg-Phe (Akil et al., 1984). In addition to these smaller enkephalin peptides, larger peptides referred to as Peptides E and F, have been isolated and contain the opioid core sequence (Mansour et al., 1995). Whereas the only source of met-enkephalin is pre-pro-enkephalin, leu-enkephalin may be cleaved from pre-pro-dynorphin as well (Calcagnetti et al., 1989). The Tyr-Gly-Gly-Phe-Leu or -Met sequence is sufficient to produce high affinity binding to the δ receptor, followed by

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moderate affinity for the μ receptor (Chavkin et al., 1981). In contrast, the κ receptor has a markedly poor affinity for Leu-and Met-enkephalin peptide core, despite the presence of three copies of Leu-enkephalin in the Pro-Dyn precursor. The C-terminal extension beyond the eight amino acid position (e.g. one that contains an arginine residue in position 6 which allows the endogenous ligand to bind to the κ receptor) destabilizes the binding of the peptide to the δ receptor , while enhancing or maintaining binding to the κ receptor (Mansour et al., 1995). Enkephalinergic perikarya are found as small interneurons at most levels of the neuraxis, including the telencephalon (cerebral cortex, olfactory tubercle, **amygdala**, hippocampus, bed nucleus of the stria terminalis and preoptic area), diencephalon (hypothalamus and periventricular and lateral geniculate nucleus of the thalamus), mesencephalon (PAG, superior and inferior colliculi and interpeduncular nucleus). met-/myelencephalon (NRM, NRGC, NTS, nucleus reticularis paragigantocellularis, lateral reticular nucleus, spinal trigaminal nucleus) and the dorsal horn of the spinal cord (e.g. Hokfelt et al., 1977; Khachaturian et al., 1983; Sar et al., 1978).

3.) Pre-Prodynorphin The pre-prodynorphin precursor is cleaved to produce three leu-enkephalin containing peptides: alpha and beta-neo-endorphin, dynorphin A and dynorphin B (Goldstein et al., 1981). Several peptide lengths of dynorphin A are biologically active, including dynorphin$_{1-8}$ (DYN B) and dynorphin$_{1-17}$ (Hollt, 1986). Several studies suggested that Dyn A_{1-8} is the minimal sequence for κ receptor affinity and emphasized importance of the arginines in the positions 6 and 7 for its κ receptor selectivity (Meng et al., 1995; Magnan et al., 1982; Oka et al., 1982). Immunoreactive

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dynorphin perikarya are found in telencephalic (cerebral cortex, striatum, **amygdala**, and hippocampus), diencephalic (suprachiasmatic, paraventricular, supraoptic and arcuate hypothalamus), mesencephalic (**PAG**), and brainstem areas (spinal trigaminal nucleus, NTS, lateral reticular nucleus), as well as in the dorsal horn of the spinal cord (Khachaturian et al., 1985).

4.) " New" Opioid Peptides

a. Nociceptin A newly discovered naturally occurring heptadecapeptide, nociceptin, proved to be an endogenous agonist of the recently-cloned orphan opioid receptor ORL_1 (Opioid Receptor-Like 1: Meunier et al., 1995). This peptide was called nociceptin because its first demonstrated physiological action appears to be increased reactivity to pain in intact animals (Reinscheid et al., 1995). The peptide's precursor is more similar to POMC which is the precursor of several unrelated bioactive peptides than pro-enkephalin and pro-dynorphin, which each contains several copies of closely-related opioid peptides (Evans et al., 1988). Meunier and co-workers (1995) found that this naturally-occurring opioid peptide agonist is endowed with pro-nociceptive properties since nociceptin shares structural similarity with dynorphin A, and its sequence is present in a larger precursor with brain mRNA expression. Synthetic nociceptin induces hyperalgesia when administered intracerebroventricularly, but can produce analgesia under certain dosing and timing conditions (Meunier et al., 1995).

b.Endomorphin Until recently, peptides that have been identified in mammalian brain are considered to be endogenous agonists for the δ (enkephalins) and κ (dynorphins) opioid receptors but none has been found to have exclusive preference for the μ receptor. Zadina and co-workers (1997) reported the discovery and isolation from brain of two

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peptides, endomorphin-1(Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2), both of which have a high affinity and selectivity (4,000-15,000-fold preference over the δ and κ receptors) for the μ receptor. These two peptides are more effective than μ-selective analogue DAMGO in vitro and produce potent and prolonged analgesia in mice They may be natural ligands for the μ receptor.

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B. OPIOID RECEPTOR SUBTYPES

Since the discovery of opioid receptors over two decades ago (Pert and Snyder, 1973; Simon et al., 1973), an increasing body of work has emerged supporting the concept of multiple opioid receptors (see reviews: Zukin and Zukin, 1981; Pasternak and Wood, 1986; Pasternak, 1993; Simon, 1993). Molecular cloning has identified several opioid receptor clones: MOR-1, DOR-1, KOR-1 and KOR-3/ORL-1(Uhl et al., 1994), confirming pharmacological and biochemical studies that previously postulated the existence of these receptor subtypes. The cloned opioid receptors are highly homologous and belong to the family of seven-transmembrane receptors (Zaki et al., 1996).

The following section will discuss μ, δ, and κ opioid receptor classes as well as their respective subtypes.

1. Mu Opioid Receptors Cells expressing mu receptor mRNA display a unique distribution, and are localized in such regions as the olfactory bulb, caudate-putaman, nucleus accumbens, lateral and medial septum, diagonal band of Broca, bed nucleus of stria terminalis, most thalamic nuclei, hippocampus, **amygdala**, medial preoptic area, superior and inferior culliculi, **PAG**, dorsal and median raphe, NRM, LC, parabrachial nucleus, pontine and medullary reticular nuclei, nucleus ambiguus, nucleus of the solitary tract, nucleus gracilis and cuneatus, dorsal motor nucleus of vagus, laminae I and II of the spinal dorsal horn, and dorsal root ganglia (Ding et al., 1996; Mansour et al., 1994). Of the three major types of opioid receptors (mu, delta, kappa) in the nervous system, the mu opioid receptor shows the highest affinity for morphine and other alkaloids of high abuse potential, as well as a variety of peptide and nonpeptide drugs that exert powerful effects

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on nociceptive, autonomic, and psychological functions (Ding et al., 1996; Raynor et al., 1995). Both presynaptic (Delfs et al., 1994) and postsynaptic localization of mu receptors have been observed (Elde et al., 1995).

a.) μ_1 opioid receptor: The μ_1 opiate receptor subtype binds opiates, most enkephalins, and beta-endorphin with similar very high affinities (see review: Pasternak et al 1993). While there are few selective agonists for the μ_1 receptor, naloxazone and naloxonazine act as irreversible and selective μ_1 antagonists (Ling et al., 1986, 1985; Hahn et al., 1982). The CXBK mouse, which displays a selective decrease in μ_1, as compared to μ_2 receptors, is less sensitive to analgesic action of i.c.v. or systemically-administered morphine without showing changes in spinal sensitivity, thereby indicating a supraspinal mechanism of action for the μ_1 receptor. Naloxonazine produces profound reductions in analgesia induced by μ agonists following systemic and supraspinal, but not spinal administration (Paul et al., 1989; Bodnar et al., 1988; Rossi et al., 1993).

b.) μ2 opioid receptor The μ_2 site selectively binds morphine-like compounds more potently than enkephalins. CTOP and β-FNA act as respective reversible and irreversible antagonists at this site, as well as at the μ_1 site (Portoghese et al., 1980). Therefore, one way of distinguishing μ_1 and μ_2 actions is to compare β-FNA and naloxonazine effects. If β-FNA(μ_1 + μ_2) and naloxonazine (μ_1) exert equal antagonist effects, the μ_1 opioid binding site would appear responsible for this action. If β-FNA(μ_1 + μ_2), but not naloxonazine (μ_1) exert effects, the μ_2 opioid binding site would appear responsible for this action. DADL and DSLET are potent δ compounds that interact quite effectively at the μ_1 opioid binding site, but show poor affinity for the μ_2 opioid

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binding site (Clark et al., 1987). The μ_2 binding site appears to possess a spinal site of action in mediating the analgesic actions of intrathecal morphine (Paul et al., 1989).

2. Delta Opiate Receptors

Mansour (1994) showed that cellular localization of delta receptor mRNA differed from mu or kappa distributions, with expression in such regions as the olfactory bulb, allo-and neocortex, caudate-putaman, nucleus accumbens, olfactory tubercle, ventromedial hypothalamus, hippocampus, **amygdala**,red nucleus, pontine nuclei, reticulotegmental nucleus, motor and spinal trigeminal, linear nucleus of the medulla, lateral reticular nucleus, spinal cord, and dorsal root ganglia. Quantitative analysis of the distribution of DOR-1 mRNA in the rat brain (Jenab et al., 1995) indicates highest levels in the caudate-putamen, followed by the frontal cortex, nucleus accumbens and olfactory tubercle. The spinal cord, **PAG** and hippocampus display moderate amounts, while the medial thalamus and cerebellum had the lowest levels. Pharmacological evidence indicates that at least two δ-opioid receptor subtypes, termed δ_1 and δ_2 respectively, may mediate analgesia in mice (Sofuoglu et al., 1991).

a.) Delta$_1$ receptor The δ_1 receptor subtype has been characterized by the agonist actions of [D-Pen2,D-Pen5]enkephalin (DPDPE) and [D-Ala2,D-Leu5] enkephalin (DADLE), and by the antagonist actions of [D-Ala2, Leu5]enkephalin-Cys6 (DALCE) and a nonpeptide opioid antagonist, 7-benzylidenenaltrexone (BNTX) (Vanderah et al., 1994; Portoghese et al., 1992). Delta$_1$ receptor appear to be involved in spinal opioid induced antinociception (Takemori and Portghese, 1993; Porreca et al., 1987: Rossi et al., 1994).

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b.) Delta$_2$ receptor The δ_2 receptor subtype has been characterized by the agonist actions of [D-Ser2, Leu5]enkephalin-Thr6 (DSLET)and [D-Ala2]deltorphinII (Stewart et al., 1994). The antagonist ligands employed to distinguish the δ_2 receptor are naltrindole 5'-isothiocyanate (5'-NTII) and the naltrindole analogue, Naltriben (NTB) (Vanderah et al., 1994; Stewart et al., 1994). However, in spite of high selectivity of these antagonists for the δ_2 receptor, high doses of NTB also possess kappa receptor agonist-like activity (Stewart at al.,1994). Further, Sofuoglu and Portoghese (1993) observed that selectivity of NTB for the delta$_2$ receptor is not maintained after s.c. administration in the rat, since it antagonized deltorphin and DPDPE to an equivalent extent. When either (Leu^5)enkephalin or (Met^5)enkephalin were administered intrathecally (i.t.), with the peptidase inhibitors, bestatin and thiorphan, the antinociceptive ED50 values of both enkephalins were significantly raised by naltriben, a selective delta $_2$-receptor antagonist, but not by BNTX, thus arguing for a spinal antinociceptive action of the delta $_2$ receptor (Takemori and Portghese, 1993).

Despite hypotheses of distinct opioid binding sites, there is also evidence for interacting binding sites, especially delta and mu complexes (Traynor et al., 1993; Xu et al., 1992). Different laboratories (Traynor et al., 1993; Cha and Rothman, 1994; Jiang et al., 1990) suggested classification of δ receptors into two subtypes, those that are either associated (called $\delta_{complexed}$ or δ_{cx}) or not associated (called $\delta_{noncomplexed}$ or δ_{ncx}) with mu receptor complexes. There is some evidence supporting the contention that δ_{ncx} and δ_{cx} are actually, synonymous with the more-widely accepted classification of δ_1 and δ_2 receptors respectively, and thereby place these two classification schemes into one

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coherent story (Cha et al., 1994). The cloned delta opioid receptor appears to be distributed primarily in axons, functions in a presynaptic manner, and appears to correspond to the δ_2 receptor subtype (Elde et al.,1995).

3. Kappa Opioid Receptors The distribution of kappa opioid receptor mRNA in adult mouse brain is also expressed in distinct areas throughout the brain, as observed using in situ hybridization histochemistry (DePaolo et al., 1994). The telencephalon has high levels of expression in the deeper layers of the parietal and temporal cortex, olfactory tubercle, nucleus accumbens, claustrum, endopiriform nucleus, nucleus of the vertical and horizontal limb of the diagonal band, and in **the medial and central nuclei of the amygdala.** In the diencephalon, kappa opioid mRNA is present in multiple medial thalamic nuclei as well as in most of hypothalamic nuclei. The mesencephalon has the highest level of kappa receptor mRNA in the substantia nigra pars compacta, ventral tegmental area, zona incerta, interpeduncular nucleus, superior colliculus, inferior colliculus, **PAG**, and the raphe nucleus. In the metencephalon, kappa opioid receptor mRNA was expressed in the parabrachial nucleus, LC, dorsal and ventral tegmental nuclei, and the raphe pontine nuclei. The fact that different κ receptor subtypes have similar affinities in different species, as well as the similarity of the cDNA sequence of human KOR to the mouse and rat sequence at the protein level, support the use of rodent models for the study of κ opioid receptors in opioid function (Simonin et al., 1994; Webster et al., 1993). Based upon the observation that the combination of nalorphine and morphine yielded a biphasic physiological response, Martin (1967) proposed the theory of Receptor Dualism which suggested two opiate receptors: M(morphine) and N (nalorphine). Martin

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then expanded this classification and proposed distinct kappa (ketocyclazocine) receptors, relative to mu (morphine) receptors, based upon physiological comparisons between morphine and ketocyclazocine in the spinalized dog (Martin et al., 1976). The kappa receptor family became increasingly important, and the dynorphins were generally acknowledged as an endogenous ligand for kappa receptors (Chavkin et al., 1981). Initial attempts to specify kappa binding sites suffered from the relatively low concentration of the kappa sites in rat brain and the relatively high affinity of the early kappa radioligands for mu and delta receptors (Kosterlitz et al., 1981). The discovery that the guinea pig cerebellum contained high levels of kappa sites with few mu or delta receptors, together with the availability of highly selective kappa agents, such as U50,488, U69,593 and norbinaltorphimine (nor-BNI), greatly facilitated studies of kappa binding.

a.) Kappa$_1$ Receptor The kappa$_1$ receptor is sensitive to the agonist actions of U50,488H, U69,593 and to the antagonist action of norbinaltrophimine (nor-BNI) (Portoghese et al., 1987; Pasternak et al., 1993). It has a spinal locus of action (Piercey et al., 1982). By examining the inhibition of [^{3}H]ethylketocyclazocine by dynorphin B and α-neoendorphin, Clark et al. (1989) observed a complex competition curve with decreased binding in a biphasic manner. Thus, two U50,488-sensitive κ_1 receptor subtypes in the guinea pig cerebellum were proposed: κ_{1a} and κ_{1b}. Dynorphin A labels both κ_{1a} and κ_{1b} receptors with very high affinity, whereas κ_{1b} receptors are far more sensitive to both dynorphin B and α-neoendorphin (Clark et al., 1989). Despite excellent affinities for κ_3 sites, ethylketocyclazocine and tifluadom also competed κ_1 binding more effectively than κ_3 binding (Clark et al., 1989).

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b.) Kappa$_2$ Receptor Kappa$_2$ receptors were established as an opioid receptor subtype entity based upon their relatively poor affinity for ethylketocyclazocine, tifluadom and cyclazocine, which contrasts greatly with κ_1 and κ_3 sites (Clark et al., 1989; Zukin et al., 1988; Tempet et al., 1988). The naltrexone analogue, IOXY has been used to label selectively κ_2 binding sites (de Costa et al., 1992), whereas [3H]Bremazocine is used to label two κ_2 sites, named κ_{2a} and κ_{2b} (Clark et al., 1989). These latter sites have markedly different ligand selectivity patterns and different anatomical distributions. The guinea pig κ_{2b} site has a high affinity for β-endorphin and DADL,a moderate affinity for [Leu^5]enkephalin (LE), DAMGO, α-neoendorphin, and dynorphin(1-8), and a low affinity for morphine, DPDPE and U58,488. In contrast, the guinea pig κ_{2a} site has a high affinity for α-neoendorphin, dynorphin(1-8), nor-binaltorphimine (nor-BNI), a moderate affinity for U50,488and U69,593 and a low affinity for LE, β-endorphin(1-31), DADL, and DPDPE (Clark, et al., 1989).

c.) Kappa$_3$ Receptor The kappa$_3$ receptor site is U50,488-insensitive and is defined by the labelling action of [^{3}H] NalBzOH in calf striatal membranes that did not correspond to κ_1 or κ_2 receptors (Zukin et al., 1988; Clark et al., 1989). In addition, nalorphine or "N"receptor agonist, exerts its analgesic action through the κ_3 receptor (Paul et al., 1991). It is the predominant opioid receptor in the brain, with density typically twice that of either mu or delta receptors (Clark and Pasternak, 1989). Kappa$_3$ analgesia is mediated supraspinally (Gistrak et al., 1989). Some compounds virtually inactive against κ_1 binding had unexpectedly high affinity for κ_3 sites, including DAMGO and Metkephamid (Burkhardt et al., 1982).

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4. μ/δ Receptor Complex Opioid receptors may exist in physically- or functionally- coupled states (Holaday et al., 1985). The antinociceptive potency of mu agonists can be positively modulated by DPDPE and negatively modulated by [Met^5]enkephalin (Horan et al., 1992; Jiang et al., 1990). Also subthreshold doses of [Leu^5]enkephalin potentiated morphine analgesia (Vaught and Takemori, 1979). In the μ/δ receptor model, it is proposed that agonists at delta receptors can modulate the action of mu agonists by changing the ability of the mu agonist-receptor complex to transduce the measured effect, yet not produce direct effects themselves alone (Vaught at al., 1982). Smith et al. (1992) and Schoffelmeer (1990) suggested that the μ/δ receptor complex is responsible for some of the actions of β-endorphin. They suggested that β-endorphin must bind at both sites to produce a physiological effect and that its action can be inhibited if either site is blocked. Thus, μ or δ agonists at subthreshold doses attenuated β-endorphin analgesia (Lee et al., 1982). Morover, μ or δ agonists, given concurrently, interact synergistically, even though δ agonists may not be active alone (Heyman et al., 1989b). Monroe and co-workers suggested three pharmacologically- distinct mechanisms which mediate the analgesic action of β-endorphin in the rat PAG. One of these mechanisms displays common characteristics with the neuronal mechanisms responsible for morphine analgesia, associated with μ opioid receptors. The remaining two mechanisms appear to be specific for β-endorphin, and incorporate receptors which are pharmacologically distinct from the defined μ, δ, κ or epsilon opioid receptors. One, in fact, appears to display no affinity for naltrexone. The same authors suggested that β-

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endorphin may activate pain facilitory mechanisms which modulate its overall antinociceptive action.

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C. ENDOGENOUS PAIN INHIBITORY SYSTEM

The pain inhibitory neuronal system in the behaving animal is part of an integrated defensive response to fear-provoking stimuli, such as an electric shock, presence of predators, stress, aggressive conspecifics, cognitive behavior or conditioned stimuli (Helmstetter, 1992; Smith et al., 1988; Bodnar et al., 1980; Terman et al., 1984; Watkins and Mayer, 1982). Research concerning descending mechanisms of endogenous pain inhibition implicated a number of discrete neuronal sites, including the midbrain PAG, the pontine LC, the RVM, as well as the number of spinal sites (Watkins et al., 1993). Opioids can modulate pain perception at both spinal and supraspinal levels of the neuraxis (see reviews: Yaksh, 1984a, 1984b). A parallel neuronal circuitry that ultimately descends to the dorsal horn acts to inhibit ascending pain transmission (Basbaum and Fields, 1984). The input to these brainstem mechanisms are initially activated by ascending nociceptive transmission, thereby forming a negative feedback loop (Basbaum and Fields, 1987). That analgesia could be conditioned or activated by stressful, but not painful environmental events suggests other more rostral inputs to the brain stem circuits in addition to the afferent nociceptive transmission itself (Good and Westbrook, 1995; Helmstetter, 1992). Thus, analgesia induced by classical conditioning (see review: Chance, 1980) is eliminated by forebrain decerebration (Watkins et al., 1982) indicating a rostral locus of activation. The amygdaloid complex is a likely participant in thias rostrally-activated circuitry. This is consistent with the amygdala's involvement in the processes of fear, anxiety, emotional memory and aggression. It also controls somatomotor, visceromotor, behavioral and neuroendocrine responses to stressors (Gray et al., 1991). Although the role of the

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amygdala in unconditioned forms of stress-induced analgesia is questionable (Pavlovic et al., 1996), its participation in conditioned analgesia is well established. There is a consensus that both unconditional and conditional analgesia converge at least at the level of the PAG (Fox, 1994; Watkins et al., 1993).

1.) The Amygdala and PAG There are numerous reports describing neuroanatomical and neurophysiological relationships between the amygdala and the PAG, therefore providing a potential substrate for the amygdala's influence upon the PAG in inducing analgesia (Da Costa Gomez and Behbehani, 1995; Beitz, 1982). The midbrain PAG is a cell-dense region surrounding the midbrain aqueduct which consists of elaborate longitudinal anatomical and functional columns (see review: Bandler, 1994). Important functions classically related to the PAG are defensive reactions, analgesia and autonomic regulation. The PAG coordinates strategies with different classes of threatening, stressful or painful stimuli which are critical to the animal's survival (Besson et al., 1991). The lateral PAG neuronal column mediates active coping strategies, that is, either confrontation or flight from a source of usually escapable threat or pain, whereas the ventrolateral PAG neuronal columns mediates a more passive coping strategy that might function to lessen the physiological and emotional impact of an inescapable stressful or painful encounter (Bandler et al., 1991). A non-opioid-mediated antinociception which is associated with active defensive behavior, hypertension and tachycardia is elicited from the lateral PAG, whereas an opioid-mediated antinociception which is associated with quiescence, hyporeactivity, hypotension and bradycardia, is elicited from the ventrolateral PAG (Lovick et al., 1991). It is interesting to observe that the amygdala which shares autonomic and homeostatic functions with the PAG, projects throughout the rostrocaudal

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extent of the PAG, most noticebly in the lateral-ventrolateral input columns (Bandler et al., 1994). The PAG, in turn, projects to theRVM, which includes the nucleus raphe magnus (NRM) and reticularis gigantocellularis (NRGC) (Basbaum and Fields, 1984). The RVM also receives input from the LC and the dorsolateral pontine tegmentum, another area implicated in nociceptive modulation, as well as from diencephalic and telencephalic structures, including the hypothalamus, the frontal cortex, **the amygdala**, and the bed nucleus of the stria terminalis (Basbaum and Fields, 1992; Holstege, 1987). Although the RVM projects to several brainstem and spinal cord sites, its major descending projections are to the spinal and trigeminal dorsal horns (Mason and Fields, 1989). Axons of the RVM terminate densely in laminae I, II, and V of the trigeminal nucleus caudalis and project via the spinal dorsolateral funiculus (DLF) to terminate in laminae I, II, V, VI, and VII of the spinal dorsal horn (Basbaum and Fields, 1992). These laminae are known to contain the terminals of small-diameter primary nociceptive afferents, as well as the neurons that respond to noxious stimuli and project to the brain stem and thalamus (Fields and Basbaum, 1978; Dubner and Bennett, 1983). There is substantial evidence that the hypoalgesia produced by activation of this endogenous pain-suppression system is attenuated or prevented by interrupting the integrity of the descending inhibitory neuronal processes, at any point (Jensen and Yaksh, 1986b).

2.) Diversity of endogenous circuitries The diversity of pain endogenous nociceptive circuitries were demonstrated by Tseng and coworkers (Tseng et al.,1984, 1985, 1989, 1993). They showed that analgesia induced by β-Endorphin and morphine are mediated by activation of different neural mechanisms. The forebrain sites sensitive to

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β-Endorphin for inhibition of the tail-flick response are located in the posterior nucleus accumbens (Acb), medial preoptic area (MPA) and arcuate hypothalamic nucleus (Arc). The brain-stem sites most sensitive to β-Endorphin that are not also sensitive to morphine, are the caudal medial medulla, including the raphe obscurus and raphe pallidus nuclei (Tseng et al., 1990). β-Endorphin microinjected into these sites putatively exerts its analgesic effect through the poorly-characterized ε opioid binding site, and is blocked by β-Endorphin$_{1\text{-}27}$, a putative ε opioid receptor antagonist (Tseng et al., 1989a). Furthermore, microinjection of β-Endorphin into forebrain sites induces the release of Met-enkephalin from the brainstem and spinal cord. Blockade of opioid receptors in the spinal cord by i.t. naloxone, β-FNA or CCK8 antagonizes analgesia induced by supraspinally-administered β-Endorphin (Tseng et al., 1985, 1989). In contrast, the forebrain sites sensitive to morphine are located in preoptic arcuate nuclei, but not in the nucleus acumbens. The brainstem sites sensitive to morphine are located in regions of the RVM (Tseng et al., 1990). At the supraspinal level, morphine acts primarily through the mu receptor (Smith et al., 1988; Fang et al., 1986; Bodnar et al., 1988). Whereas i.c.v. or i.c. morphine analgesia is blocked by i.c.v. or i.c. naloxone,β-Endorphin analgesia is unaffected (Tseng et al.,1989). At the spinal level, supraspinally-administered morphine activates the descending noradrenergic and serotonergic systems to produce analgesia, and blockade of alpha$_2$ adrenoceptors in the spinal cord by i.t. injection of yohimbine or phentolamine or by injection of serotonergic receptor antagonist methysergide, antagonizes morphine, but not β-Endorphin-induced analgesia (Tseng et al., 1984, 1985, 1989, 1992b). The dissociation of these two systems is further shown by the lack of

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cross-tolerance , and only additive analgesia following ventricular and intrathecal administration of subthreshold doses of each agonist (Tseng et al., 1990, 1993). Finally, pentobarbital anesthesia blocks supraspinal morphine analgesia, but not β-Endorphin analgesia (Tseng et al., 1992a).

3.) The Neurophysiology and Neurochemistry of Pain-Inhibitory Circuits

Three physiologically distinct classes of neurons can be identified in the RVM based upon the temporal correlation of changes in their firing with the execution of nocifensor reflexes elicited by noxious stimulation: "on-cells", "off-cells" and "neutral cells" (Fields et al., 1991). The "on" and "off-cells" play a central role in descending nociceptive modulation . Both cell classes are excited by electrical stimulation in the PAG at a current sufficient to inhibit the tail-flick and the target of their action is likely to be the dorsal horn (Mason and Fields, 1989). In their basic model of RVM intrinsic circuitry, Fields et al. (1991) suggested that the "off-cells" have widespread connections, exciting other "off-cells" and providing an anatomical substrate for the antinociceptive effect. The "off-cells" and "on-cells" are mutually inhibitory to each other. The "off-cells" exert a net inhibitory effect on nociceptive transmission and its pause in firing (e.g. 400ms prior to the tail-flick response) permits nociceptive transmission and execution of nocifensor reflexes (Fields et al., 1991). If the onset of the "off-cell" pause is advanced by noxious stimulation applied at the distant body site, just prior to the noxious heating of the tail, TF latencies decrease (Ramirez and Vanegas, 1989). The "off-cell", and no other RVM neurons, become continuously active following administration of morphine either systemically (Fields et al., 1983b) or by microinjection into the PAG (Cheng et al., 1986) in doses sufficient to inhibit

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the TF. On the other hand, the "on-cells" are highly active just prior to and during execution of the TF response, indicating that they do not have a potent inhibitory action on nocifensive reflexes. There is actually evidence for their facilitatory role in nociception. Administration of morphine suppresses "on-cell" firing (Cheng et al., 1986). During the period of reversal of morphine's antinociceptive action by an opiate antagonist, there is a significant enhancement of nociceptive responsiveness associated with a high rate of RVM "on-cell" firing (Bederson, 1990) that can be attenuated or reversed by microinjection of a local anesthetic into the RVM (Kaplan and Fields, 1990). The third kind of cells that display no change in firing related to the execution of the TF response are termed "neutral cells", and appear to play minor roles in descending pain inhibition.

Because the direct cellular actions of opioid receptor agonists are generally regarded to be inhibitory, opioid excitation of "off-cells" is likely due to disinhibition (Fields et al., 1983). Consistent with this is the observation that enkephalin-immunoreactive axonal swelling are apposed to intracellularly-labeled RVM "on-cells" (Back et al., 1990). Since GABA mediates the RVM "off-cell" pause, it is suggested that a subset of RVM "on-cells" are GABAergic inhibitory interneurons (Pan and Williams, 1990). One putative "off-cell" neurotransmitter is serotonin. Microinjection of 5HT into the RVM has an antinociceptive action (Aimone and Gebhart, 1986). Systemic morphine increases the concentration of 5HT metabolites in the RVM (Rivot, 1989). Also, depletion of spinal cord 5HT by the neurotoxin 5,7-dihydroxytryptamine blocks the antinociceptive effect of morphine microinjected into the RVM (Vasko et al., 1984). Given the fact that the "off-cells" are the only RVM neurons activated by the opioid administration, and considering the above-

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mentioned observations about serotonin function, it appears that, at least, part of the "off-cells" contain 5HT. Significant extrinsic sources of 5HT to the RVM are neurons in the PAG and the midbrain B8 and B9 cell groups (Beitz et al., 1983). In addition to serotonin, the pathway from the PAG area to the RVM also contains EAA, Neurotensin, Somatostatin, Substance P and Norepinephrine (Wiklund et al., 1988; Baitz, 1983; Takagi et al., 1981). Since microinjection of EAA antagonists into the RVM significantly increased the PAG-stimulating current required to inhibit tail-flick latencies, Fields et al. (1991) suggested that the EAA-containing afferents from PAG produce antinociception by exciting RVM "off-cells". In this regard, morphine analgesia elicited from the PAG is reduced by NMDA, and to a lesser degree, non-NMDA antagonists administered into the RVM (Spinella et al., 1996; vanPraag and Frenk, 1990). Neurotensin also has a modulatory role and, when microinjected into the RVM produces a dose-related suppression of TF latencies(Fang et al., 1987) and alters morphine analgesia (Urban and Smith, 1993, 1994). Norepinephrine, through α_1 and α_2 receptors, exerts respective facilitatory and inhibitory effects on "on-cells"and therefore, decrease and increase, respectively, TF latencies (Heinricher, MM and Haws, CM, 1992). Noradrenergic input to the RVM derive, in part from A5 and A7 in the pons, as well as from the A1 catecolamine cell group in the ventrolateral medulla (Kwiat and Basbaum, 1992; Dong and Shen, 1986).

4.) The final destination: Possible circuits at the level of the dorsal horn

Fields et al. (1991) suggested the following several possible circuits for RVM inhibition of dorsal horn nociceptive transmission: First, the RVM "off-cell" serotonin-containing axons

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exert inhibitory actions on thalamic projection neurons through an interaction with norepinephrine-containing axons. Second, the RVM "off-cells" directly inhibit nociceptive dorsal horn cells. Third, the RVM "off-cell" axons inhibit the nociceptive projection neuron indirectly, through the inhibition of excitatory interneuron in the dorsal horn. Fourth, nociceptive transmission cells in the dorsal horn are inhibited by dorsal horn interneurons in lamina I, which may contain either enkephalin or GABA. These inhibitory interneurons are activated by RVM "off-cell" axons, some of which contain serotonin and some of which do not.

5.) Functional Interrelationship between Opioid sites in the Neuraxis Two ways to investigate functional relationships between two sites in analgesic processes are to attenuate or block analgesia induced at one site by destruction or application of appropriate antagonists to a second site, and to study site/site and/or drug/drug interactions between sites.

The first approach has yielded the following results: Morphine analgesia elicited from the PAG could be either attenuated or blocked by different opioid and non-opioid antagonist microinjected into the RVM. Serotonergic receptors in the RVM participate in the mediation of morphine analgesia elicited from the PAG since either general (methysergide), $5HT_{2a}$ (ritanserin) or $5HT_3$ (ICS205930) antagonists reduced mesencephalic morphine analgesia on both nociceptive tests (Kiefel et al., 1992a,b). Opioid receptors in the RVM also modulate mesencephalic morphine analgesia since either general (naltrexone), μ (β-FNA) or δ (naltrindole) antagonists reduced mesencephalic morphine analgesia on both tests (Kiefel et al., 1993). Mesencephalic morphine analgesia

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was also markedly reduced on both tests after RVM pretreatment with either competitive (AP7) or noncompetitive NMDA antagonist, MK-801. Small but significant reductions in mesencephalic morphine analgesia occured only on the jump test following a kainate/AMPA antagonist, CNQX(Spinella et al., 1995). In contrast, a selective neurotensin antagonist in the RVM enhances mesencephalic morphine analgesia on the tail-flick test (Urban and Smith, 1993). These antagonistic effects in the RVM were quite selective since the antagonists failed to alter baseline nociceptive latencies or thresholds. Further, medullary cannula placements that were lateral, dorsal or ventral to the RVM failed to antagonize mesencephalic morphine analgesia (Kiefel et al., 1993). In contrast, Spinella (Spinella et al., 1997) have shown that M1 (pirenzepine) and M2 (methoctramine)receptor antagonism reduced mesencephalic morphine analgesia only on the jump test in sites both within and surrounding the RVM. Also, the nicotinic receptor antagonist, mecamylamine significantly reduced mesencephalic morphine analgesia on both tests in sites both within and surrounding the RVM. In contrast to the selective actions of serotonergic, opioid and excitatory amino acid receptor antagonists in the RVM, these data suggest that the supraspinal mediation by cholinomimetic drugs of mesencephalic morphine analgesia utilizes a number of ventral medullary structures in addition to the RVM. The above-mentioned studies implicated the importance of serotonergic, opiate, kainate-quisqalate, NMDA and neurotensin receptors in the RVM in the modulation of mesencephalic morphine analgesia, thus delineating some neurochemical PAG-RVM relationships in the modulation of the analgesic processes.

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A second approach to examine drug-drug and/or site-site interactions uses analgesic synergy studies as a model. Synergy is operationally defined as a greater than additive effect when subthreshold doses of an agonist are applied to two sites. Yeung and Rudy (1980) found that concurrent i.t. and i.v.t. injection of morphine interacted in a supra-additive manner to induce analgesia, and that the strength of this mutual potentiation was apparently greatest at a 1: 1 ratio of supraspinal to spinal dosage. At low to moderate systemic morphine doses, both the spinal and supraspinal substrates have an obligatory involvement in mediating the analgetic effect, whereas at high systemic doses, spinal and supraspinal agonisms become capable of mediating analgesia by independent actions. A series of studies (Bodnar at al., 1991; Rossi et al., 1993, 1994) have shown that subthreshold doses of morphine which are analgetically inactive alone, will produce potent analgesia when simultaneously coadministered into combinations of three regions: PAG and RVM, PAG and LC and RVM and LC (Rossi et al., 1993). The most sensitive interactions occured for subthreshold morphine doses in the PAG and RVM, which was completely blocked by the μ_1 antagonist, naloxonazine. A second study (Bodnar et al., 1991) found that microinjection of the putative κ_1 agonist ethylketocyclazocine, or U50488H into either the PAG or LC alone failed to produce analgesia. However, coadministration of ethylketocyclazocine, but not U50488H into the PAG and LC produced a robust, naloxonazine-sensitive analgesia, implying the μ_1 receptor in this response. This particular study implicated the importance of regional analgesic interaction, as opposed to condition in which only one microinjection site is utilized.

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It was also shown that coadministration of a low-analgesic dose of the selective μ agonist, DAMGO, potentiates the analgesia produced by either the selective δ agonist, DPDPE, or the selective κ agonist, U50,488H given intrathecally (i.t.) (Sutters et al., 1990). Intrathecal δ and κ opioid agonists administered simultaneously, as well as μ and δ opioid receptor agonists administered to spinal and supraspinal sites each produse synergistic and supraadditive effects (Heyman et al., 1988). Rossi and coworkers (Rossi et al., 1994) found that subthreshold doses of DAMGO coadministered simultaneously into the PAG and RVM produce a multiplicative analgesic interaction, implying μ/μ synergy. An interaction was also observed when DAMGO (μ agonist) was applied to one site and deltorphin II (δ_2 agonist) was applied to the second site, but not when DAMGO (μ agonist) was applied to one site and either U50488H (κ agonist) or DPDPE(δ_1 agonist) was applied to the second site. These data indicate the presence of μ/δ_2, but not μ/δ_1 or μ/κ_1 interactions.

Smith (1992) observed the occurrence of synergism when subanalgesic doses of β-endorphin and morphine are coadministered into the PAG. Using different μ opioid antagonists, he suggested that β-endorphin and morphine may function through separate and distinct receptor systems that may exist on separate inhibitory neuronal processes. Another possibility is that both of these agonists activate a single descending pathway, by actions through a receptor complex with multiple sites for differential agonist binding (Monroe et al., 1996). No matter what the different mechanisms for β-endorphin and morphine could be, a common theme in the synergy mechanism is that agonists ultimately

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have to functionally converge, either at supraspinal levels or at the level of dorsal horn neurons.

At the present time, the mechanism underlying analgesic synergy is unknown. Different lines of evidence suggest different explanations. For example, opioid agonists can act at different receptor sites, and that these two populations of opioid receptors, on the same or different neurons, interact to enhance each other's activity and produce a synergistic analgesic interaction. Consistent with this is the finding that demonstrated the presence of both μ and δ-opioid receptors on primary nociceptive afferents and on second-order dorsal horn neurons in the rat (Fields et al., 1980). Also, δ, μ and κ receptors were observed on a single dorsal root ganglion (DRG) cell in vitro (Werz et al., 1987). Pick and Pasternak (1992) demonstrated that i.c.v. morphine potentiates a fixed low dose of i.t. morphine as effectivelly in μ_1-deficient CXBK mice as in the regular CD-1 mice, in spite of the fact that CXBK mice are insensitive to systemically administered morphine. The μ_1-selective antagonist naloxonazine does not diminish the potency of i.c. v. morphine in the synergy model, but it blocks morphine analgesia following supraspinal administration alone. In contrast, β-FNA which blocks both μ_1 and μ_2-opioid receptors diminishes the potency of i.c.v. morphine when administered alone or when paired with i.t. morphine. Therefore, μ_2 receptors mediate both spinal analgesic responses and the synergism with spinal systems at the supraspinal level. Thus, the involvement and functional efficacies of receptors activated by certain combinations of ascending and descending pathways in the synergy model- that are dorment when only one route of administration, one drug or one site is activated- could very well explain the supra-additive effect of simultaneous

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application of drugs. Some authors (Siuciak and Advokat, 1989; Fujimoto et al., 1988) suggested that the antinociceptive effect of i.t. morphine is tonically suppressed by descending inhibitory input. Supraspinal morphine removes this descending inhibition and allows the antinociceptive effect of spinal morphine to be expressed. This explanation is incompatible with the common view that supraspinal morphine increases descending inhibition of spinal nociceptive processing.

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D: THE AMYGDALA

1. The Neuroanatomy of the Amygdala

Because of the complexity of the primate amygdala, meager anatomical attention and lack of standardazed nomenclature, the literature on amygdala nuclei and their intrinsic and extrinsic connections use multiple terminologies. Amaral (Amaral et al., 1992) has provided one of the most detailed delimitations and subdivisions of amygdala nuclei. His description of the intrinsic organization of the amygdala begins with the deep nuclei (e.g. lateral, basal and accessory basal), followed by the superficial nuclei and areas (e.g. anterior cortical nucleus, medial nucleus, nucleus of the lateral olfactory tract, peryamygdaloid cortex, posterior nucleus), followed by the central nucleus and the remaining amygdaloid nuclei (e.g. anterior amygdaloid area, amygdalohippocampal area and intercalated nuclei). Based on the neuronal morphology, amygdaloid nuclei are categorized into: **1.** cortex-like nuclei (basal, lateral, accessory basal, periamygdaloid, cortical and amygdalo-hippocampal area nuclei) which are nearer the brain surface and exhibit pyramidal or modified pyramidal neurons, and **2.** noncortex-like nuclei (central and medial nuiclei) which do not contain pyramidal-like neurons (DeOlmos et al., 1987). The amygdaloid complex is generally considered to have two major extrinsic fiber systems: **the ventral amygdalofugal pathway** and **the stria terminalis** which are primarily subcortical. However, they do not carry distinct bundle of fibers, and fibers from one bundle appear to join fibers in the other bundle throughout their trajectories. Both the amygdalofugal and amygdalopetal connections travel through the external capsule, which is laterally and ventrally adjacent to the amygdaloid complex, and connect with the

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neocortex. The amygdala has extensive and reciprocal connections with olfactory, visual and auditory cortices, the basal forebrain, limbic and polysensory cortices, diencephalon, striatum and hippocampal formation (Ragsdale and Graybiel, 1988; Hellendall et al., 1986; Price and Amaral, 1981, 1984; Price et al., 1987; Witter and Amaral, 1991; Price, 1990; Friedman, 1986, Calderazzo et al.,1996; Aggleton, 1993; Bacon et al., 1993; Amaral et al.,1992).

Fibers from the central nucleus of the amygdala (CNA) descend into and through the midbrain, pons and medulla with some extensions through cervical levels of the spinal cord (Price and Amaral, 1981). Throughout their course the fibers innervate a number of structures that have been implicated in autonomic control and somatomotor and autonomic responses to stress. In the midbrain, the central nucleus projects to the ventral tegmental area and substantia nigra. Caudal to this, the projection extends into peripeduncular nucleus , tegmental reticular formation and the PAG. In turn, the rostral half of the PAG projects back to the CNA, however these two-way projections from the PAG and the CNA, do not appear to share point-to-point reciprocity (Bandler et al., 1994). The central nucleus projection continues through the pontine reticular formation and terminates heavily in the parabrachial nuclei located around the superior cerebellar peduncle. A few of these fibers extend medially into the NRM (Price and Amaral, 1981). Neurons in the CNA also project to the central portions of the LC containing primarily noradrenergic somata and proximal dendrites, and ramify extensively in the pericoerulear region where they occasionally form inhibitory-type synapses with cathecolamine, but mainly non-catecholamine-containing dendrites (VanBockstaele et al., 1996). However,

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there is a prominent distribution of CNA efferents to the dorsolateral pontine tegmentum adjacent to the superior cerebellar peduncle which contain noradrenergic dendrites of the LC as well as abundant non -cathecolaminergic neurons (Aston-Jones et al., 1991). Since the LC is an important region for arousal mechanisms, alertness, attention, pain and analgesia, processed information conveyed to the LC from the amygdala may help integrate behavioral and visceral responses to threatening stimuli and can bring about adaptive behavioral responses by influencing the widespread noradrenergic projections from the LC. In the medulla, the amygdala projection is distributed in the lateral part of the reticular formation, most heavily to the nucleus of the solitary tract and the dorsal motor nucleus of the vagus (Price and Amaral, 1981). An anterograde tracing study of Danielsen and Grey (1988) have shown that the central nucleus of the amygdala is reciprocally connected to the dorsal vagal complex which consists of nucleus of the solitary tract (nTS) and the dorsal motor nucleus of the vagus. This in turn joins with other preganglionic parasympatethic regions (e.g. nucleus ambiguus) as well as to areas that directly or indirectly influence preganglionic sympathetic activity. The innervation of the nTS and the dorsal motor nucleus of the vagus nerve by the central nucleus of the amygdala provides an anatomical substrate for the amygdala's modification of heart rate, blood pressure, influence on gastrointestinal motility and secretion of gastric acid, insulin and glucagon, respiratory changes and amygdala's role in eliciting behavioral responses (Laughton et al., 1987; Petrov et al., 1995).

2. The Neurochemistry of the Amygdala

a.) Monoamines The monoamines exert profound influences on the functions of

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the amygdala which receives input from four major monoamines sources. The dopaminergic innervation of the amygdala arises from the dopamine-containing cell bodies in the substantia nigra pars compacta (SN; A9 cell group), ventral tegmental area (VTA; A10 cell group) and caudal extension of the SN (A8 cell group) in the ventral midbrain. Many of the SN-VTA dopaminergic neurons innervating the amygdala also contain the peptide cholecystokinin (Seroogy et al., 1989) and a small portion of these neurons also provides a neurotensin-containing input to the amygdala (Seroogy et al., 1987). The great majority of dopaminergic axons projecting to the amygdala enter at all levels of the ventralamygdalofugal/ansa peduncularis pathway (Fallon and Loughlin, 1982). Fallon (1978) found that majority of noradrenergic innervation of the amygdala arises bilaterally from the locus coeruleus (A6 cell group) and many fibers share the same ventral amygdalofugal pathway as the dopaminergic fibers. The serotonergic innervation of the amygdala arises from the midbrain and pontine raphe nuclei (Petrov et al., 1993). This projection is unilateral and particularly dense innervation is present in the magnocellular and parvicellular parts of the basal nucleus, medial edge of the central nucleus, medial nucleus and the central nucleus (van der Koy and Hattori, 1980). Distribution of epinephrine in the amygdala is generally very sparse and localized in a few nuclei (Mezei, 1989). Overall, the densest monoamine inputs to the amygdala are in the medial sector of the central nucleus, where all four monoamines are concentrated (Fallon et al., 1982). Other amygdaloid nuclei receive a variable concentration of input from monoaminergic neurons (Fallon et al., 1987). The amygdala is different from other forebrain regions in that monoaminergic neurons projecting to this region are not highly collateralized,

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suggesting a unique monoaminergic regulation of amygdala functions (Fallon and Ciofi, 1992).

b.) Neuropeptides The amygdala has a variety of cells and fibers which contain one or some of virtually every neuropeptide found in the brain and is the most peptide-rich region in the brain outside of the hypothalamus (Price et al., 1987). The central nucleus of the amygdala is rich in Substance P, Vasoactive Intestinal Peptide (VIP), Neurotensin (NT), Galanin (GAL), Somatostatin (SOM), Corticotropin-Realising Factor (CRF), and Enkephalin (Cassel and Gray, 1989). The ENK, NT, SOM, and CRF neurons in lateral and lateral capsular subdivisions of the central nucleus appear to be best classified as medium spiny neurons on the basis of the available perikaryal and dendritic morphology (Cassel and Gray, 1989). Enkephalin-immunoreactive neurons are prevalent in the BST and central nucleus, but are for the most part confined to their dorsal and lateral subdivisions, respectively. Transection of the stria terminalis results in a reduction of GABA and enkephalin within the BST and central nuclei. It may be that enkephalin/GABA neurons are interneurons that provide a functional link between the central nucleus and BST (LeGal LaSalle and Ben-Ari, 1981).

The basolateral amygdaloid complex is rich in Vasoactive Intestinal Peptide (VIP), SOM and Cholecystokinin (CCK) (McDonald, 1985a). At least 80% of the neurons in the lateral and basolateral amygdaloid nuclei that possess SOM or NPY immunoreactivity also contain GABA (McDonald and Pearson, 1989). Since there is also extensive coexistence of GABA, SOM, NPY in nonpyramidal neurons of the cerebral cortex, these findings support the idea that the basolateral amygdala shares many important features with the

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cortex (Hendry et al., 1984a,b; Vankova et al.,1996). Destruction of the bed nucleus of the stria terminalis resulted in a prominent bilateral increase in the number of neuronal perikarya immunoreactive for [Met]enkephalin in the lateral/basolateral amygdaloid complex, again suggesting that the BSTplays an important role in the regulation of the enkephalin gene expression in the BL nuclei of the amygdala. Pu et al. (1994) observed that repeated administration of morphine increased CCK gene expression in the amygdaloid complex with the most heavily labeled neurons in lateral, basal and cortical nuclei. He suggested that the development of tolerance to morphine analgesia is due, in part, to a compensatory increase in CCK synthesis and/or release in the amygdaloid complex.

3. The Amygdala and its role in Fear

One of the first clues about the amygdala's role in fear processes was derived from the classic studies of Kluver and Bucy (1937). They found that bilateral temporal lobe resections in monkeys produced an eclectic deficit (e.g. Kluver-Bucy syndrome) that was characterized by visual agnosia, hypersexuality, reduced neophobia, and loss of fear, later proved to be due specifically to the damage in the amygdala (Weiskrantz, 1956). Consistent with this, a subsequent study showed that lesions placed in the cortical or central nuclei of the amygdala reduced emotionality in wild rats, and increased the number of contacts that a rat would make with a sedated cat (Kemble et al., 1990). Lesions placed in the amygdala either completely blocked or attenuated innate as well as conditioned reactions to stress (Davis,1994). As compared to lesion studies, electrical or chemical stimulation of the amygdala elicits feelings and behaviors related to fear and anxiety, and

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autonomic reactions indicative of fear (Gloor et al., 1981). All of these effects of stimulation are produced in the absence of any prior explicit fear conditioning, arguing that this behavior pattern had been "hard-wired" during evolution.

Papez (Papez, 1937) suggested that signals from sensory organs travel to the thalamus, from which they are distributed to the cortex (setting up a "stream of thought"), the basal ganglia (setting up a "stream of movement"), and the limbic system (setting up a "stream of emotions") that determines the respective cognitive, motor and emotional significance of the sensory input. Of all areas in the CNS, **the amygdala** is most clearly implicated of the evaluation of the emotional meaning of incoming sensory stimuli. It is also essential for the conditioning of fear responses (Le Doux, 1986) and for the establishment of associations between sensory modalities. The role of the amygdala in the processes of fear and anxiety support suggestions that the human amygdala functions to attach fearful or anxious elements to neutral stimuli associated with trauma as described in post-traumatic stress disorder (Charney et al., 1993). McGaugh (1992) suggested that the degree of arousal produced by the unconditioned stimulus, and not its aversive nature per se, determined the level of involvement of the amygdala in aversive conditioning paradigms. Thus, the amygdala appears critical for conditioning that involves an obvious fear component such as that produced by aversive shocks. Consistent with this finding, subsequent work (Davis et al., 1994; Fanselow, 1994; LeDoux, 1994 and 1986) delineated the amygdala's role in the acquisition of conditioned aversive learning. It is now apparent that, within the amygdala, these are two systems with specific roles in regard to fear conditioning.

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The basolateral complex, consisting of the lateral (LA), basolateral (BL), and basomedial (BM) amygdalar nuclei, is a substrate for the sensory convergence from both cortical and subcortical areas, and is a necessary locus for CS-US associations during fear conditioning. On the other hand, the central nucleus of the amygdala, which receives input from the basolateral nucleus and projects to such brain areas as the lateral hypothalamus (LH), PAG, parabrachial nucleus (PBN), ventral-tegmental area (VTA), dorsal motor nucleus of vagus, pontine reticular formation, trigeminal and facial motor nuclei and paraventricular nucleus of the hypothalamus (PVN), is considered to be a final common output pathway for the generation of fear CRs. "The fear-potentiated startle effect" is one of the paradigms frequently used to demonstrate the amygdala's role in conditioned fear. Conditioned fear is operationally defined by an elevated startle amplitude in the presence, relative to the absence, of a cue previously paired with a shock. Davis (1991) found a direct connection between the central nucleus of the amygdala and a precise locus in the nucleus reticularis pontis caudalis critical for the startle reflex. Electrolytic lesions placed along this output pathway completely block the development of fear-potentiated startle, whereas lesions placed in the other major output of the amygdala through the stria terminalis and bed nucleus of the stria terminalis do not. Davis (1992) indicated that lesions placed in the central amygdaloid nucleus totally blocked initial acquisition of fear-potentiated startle, but that the same lesions fail to prevent re-acquisition. Thus, it appears that the central nucleus of the amygdala induces functional changes in a secondary brain system during initial training, or alternatively plays a

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permissive role to allow this secondary system alone to support fear-potentiated startle when the central nucleus is subsequently removed or inactivated.

4. The Amygdala and its role in Anxiety

Kopchia (1992) suggested that the central nucleus of the amygdala plays an important role in maintaining a tonic level of anxiety-like behavior, and that a lesion placed in this nucleus results in anxiolytic-like effects in many conflict paradigms and animal models of anxiety. Graeff et al. (1992) suggested that the main anatomical substrates of fear and anxiety are represented by areas of the amygdala and hypothalamus at its rostral pole, and the midbrain PAG at its caudal pole. They found that brief exposure (15 min.) to an elevated plus-maze, an ethologically based animal model of anxiety, leads to the appearance of c-fos immunoreactivity (e.g. an early oncogene marker of neuronal activation) in the pyriform cortex that sends massive sensory input to the amygdala, **in several nuclei of the amygdaloid complex**, in the periventricular system-along the antero-posterior extension of the hypothalamus, and in its prolongation to both the midbrain tectum/dorsal PAG, the paraventricular thalamus and the LC. This longitudinally-organized and integrated circuitry in the brain commands defensive behavior and elaborates aversive emotional and motivational states. Fanselow (1991) suggested that the function of the amygdala is to synthesize various stimulus inputs from the environment, and then signal to the PAG the degree of threat they represent to the organism. If the context of the threat requires elaborate assessment, then the amygdala activates and acts in concert with the hippocampus. The PAG selects, organizes and commands the appropriate behavioral and neurovegetative defensive reactions. Stressful conditions, including threat, activate 5-HT

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neurons in the raphe nuclei, thus increasing 5-HT release (Kalen et al., 1989). This activation is likely to be mediated by the amygdala, since it is abolished following bilateral lesions placed in the central nucleus (Singh et al., 1990). Deakin and Graeff (1991) suggested that the combined actions of 5-HT-mediated facilitatory actions in the amygdala and 5-HT-mediated inhibitory actions in the PAG allow for learned escape or avoidance strategies to take precedence over less flexible, panic-like behavior. Only when the intensity of threat becomes too high, 5-HT inhibition of the PAG is overcome, and phylogenetically primitive reactions, such as "blind" fight-flight and defensive agression, are expressed. Thus, phenomenologically, one would say that fear or anxiety act to inhibit panic. According to this view, the amygdala would be mainly responsible for conditioned fear and anticipatory anxiety. Dysfunctional activation of these mechanisms would result in the morbid anxiety state observed in human beings, known as generalized anxiety disorder (GAD). In contrast, the PAG would organize the response to unconditioned aversive stimuli, such as pain, asphyxia and innate fear-inducing stimuli (predators' odor or sight). Clinically, its dysfunction would result in panic disorder and phobias. Graeff and co-workers (1992) have shown that microinjection of either benzodiazepines (BDZ), a GABA-A receptor agonist or a 5-HT receptor antagonist into the amygdala has anxyolitic effects in conflict tests and other models of conditioned fear and anxiety, thereby increasing the exploration of the open arms of the elevated plus-maze. Similar administration of either 5-HT or 5-HT1A receptor agonists has anxiogenic effects in the amygdala. In contrast, electrical strimulation of the PAG or the PAG microinjection of 5-HT itself, 5-HT mimetics, or drugs (e.g. BDZ or GABA agonists) that enhance the action

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of endogenous 5-HT has an antiaversive effect. Further, microinjection of either midazolam, the NMDA receptor antagonist AP-7, or the B-adrenergic receptor blocker, propranolol increased exploration of an elevated maze, thereby producing an anxiolitic effect. These results taken together point to an inhibitory role of the GABA-BDZ system in both the amygdala and the PAG. In contrast, 5-HT apparently enhances conditioned fear in the amygdala, while inhibiting unconditioned fear in the PAG. Thus, a 5-HT2/1C antagonist in the amygdala released punished behavior, yet antagonized the anti-aversive effect of 5-HT, zimelidine and 5-HT1A/1B receptor blockers in the PAG. Davis, Rainnie and Cassell (1994) suggested that treatments that increase the excitability of amygdala output neurons in the basolateral nucleus by decreasing opiate and GABA transmission, and increasing noradrenergic transmission, improve aversive conditioning. In contrast, treatments that decrease excitability of these neurons, by increasing opiate and GABA transmission, and decreasing NMDA and noradrenergic transmission, retard aversive conditioning in addition to producing anxiolytic effects in appropriate animal tests. Liebsch and co-workers (1995) have shown that corticotropin-releasing hormone (CRH) microinjected to the central nucleus of the amygdala is critically involved in the mediation and expression of anxiety-related behavior. Microinjection into the central nucleus of the amygdala of an antisense oligodeoxynucleotide corresponding to rat CRH1 receptor mRNA reduced anxiety-related behaviors as measured on the plus-maze after social defeat stress. Although increased anxiety may lead to to an enhanced tendency to avoid social contact, this procedure did not affect the performance of rats on the social discrimination

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test, thus suggesting that an involvement of CRH receptors in the CeN in this model of olfactory short-term memory is unlikely.

5. The Amygdala and its role in Aggression

Aggression in humans apparently decreases with damage in the amygdala and increases with seizure activity in the amygdala (Potegal et al., 1996; Albert et al., 1993). According to some authors, human aggression has its biological roots in the defensive aggression of nonprimate mammals. There is a high correlation of increased attack probability in animal models of aggression with neural activation within the corticomedial amygdala (Potegal et al., 1996). Adrenergic activation of the corticomedial amygdala with testesterone microinjection increases aggression and, following a single aggression test, c-Fos was significantly increased only within the corticomedial amygdala (Joppa et al., 1995; Matochik et al., 1994). Also, damage within proximal amygdaloid nuclei disinhibits the central nucleus, and encourages aggression (Desjardins and Persinger, 1995). The significant decrease of histamine levels in the amygdala with simultaneous activation of the noradrenergic system suppresses muricide (e.g. mouse-killing aggression) in rats brought about by a thiamine-deficient diet (Onodera et al., 1993). Although defensive rage is not elicited from the amygdala, the pathways that arise from different nuclei of the amygdala appear to possess modulatory roles in defensive rage behavior (Siegel and Schubert, 1995; Shaikh and Siegel, 1994). Two pathways from the basal and medial amygdala that facilitate defensive rage have been identified. The first arizes from the basal amygdala and projects directly to the PAG acting upon NMDA receptors in the PAG. The second arises from the medial amygdala and projects to the medial hypothalamus using substance P and

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acts through NK1 receptors in the medial hypothalamus. The latter pathway functions to supress predatory attack behavior elicited from the lateral hypothalamus, and this effect is achieved by an inhibitory pathway from the medial hypothalamus that innervates the lateral hypothalamus. A third modulatory pathway arises from the central nucleus of the amygdala and projects to the PAG. Its powerful suppressive effects upon PAG-elicited defensive rage behavior are mediated through opioid peptides that act upon mu receptors within the PAG. Significant reduction of noradrenaline (NA) followed by decreases in dopamine and serotonin, secondary to NA depletion in the amygdala, result in decreases in defensive episodes and marked increases in offensive aggression (Zagrodzka et al ., 1994).

6. The Amygdala and its role in Emotional Learning and Memory

LeDoux (1993) used fear conditioning, the neural and cellular mechanisms of which are relatively well known, to describe the amygdala's role in emotional learning. However, he observed that challenges of this approach include needs to convert the proposed fear conditioning system into a more general understanding of emotional memory, including the learning of more complex kinds of fears, as well as responses to other emotions. The neural pathways involved in the association of an auditory CS with a footshock US have been identified. Lesions placed in the auditory thalamic nuclei completely eliminate the ability of an animal to associate an auditory CS with a shock (US). In contrast, lesions placed in the auditory cortex have no effect on conditioning, but lesions placed in the amygdala prevent this form of conditioning (Davis et al., 1992). LeDoux observed that thalamo-amygdaloid projections are involved in conditioning to simple, undiscriminated modality-specific inputs, and this pathway is unable to distinguish

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between the two stimuli, such as in discriminated conditioning tasks in which one CS is paired with a US and the other is not. In contrast, cortico-amygdala projections, such as primary sensory neocortex-unimodal association cortex-lateral amygdaloid nuclei, are required for conditioning to stimuli that must be discriminated on the basis of its perceptual feature. Hippocampo-amygdala projections have been implicated in contextual conditioning. Connections between complex association cortex (e.g. multimodal areas of the frontal lobe) and the amygdala appear to be involved in the formation of emotional memories on the basis of complex cognitions. LeDoux emphasized the following points:

1. The amygdala is provided with various levels of CS representations as well as US representation and this makes conditioning possible by modifying the neural processing in any or all of the various levels of CS representation.

2. Thalamo-amygdalo input is glutamate-containing terminating on projection neurons (containing NMDA and AMPA receptors) that give rise to excitatory projections to target areas, but also through local collateral projections to GABA inhibitory interneurons. One way in which the unconditioned stimulus (US) might mediate conditioning is by removing the influence of the inhibitory interneuron in the recurrent collateral feedback loop. This would allow the CS to exert a stronger excitatory impact on target areas.

3. The amygdala is reciprocally interconnected with the hypothalamus and basal forebrain systems involved in motivational controls. Basal motivational states may have important influences on emotional system functioning. For example, a hungry animal may be less fearful than the satiated one. Van Der Kolk (1994) suggested that moderate to

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high activation of the amygdala enhances the long-term potentiation of hippocampally-mediated declarative memory which may account for hyperamnesias of a stressful experience. Excessive stimulation of the amygdala interferes with hippocampal functioning , inhibiting cognitive evaluation of experience and semantic representations (Van der Kolk, 1992). Memories are then stored in sensorimotor modalities: somatic sensations and visual images. These emotional memories are thought to be relatively indelible, but their expression can be modified by feedback from the prefrontal cortex. Van Der Kolk also suggested that the amygdala integrates internal representations of the external world in the form of memory images with emotional experiences associated with those memories. Answering the question as to whether aversive memories are actually stored in the amygdala, Liang (1991) has shown that retention of an inhibitory avoidance response in rats was impaired when the amygdala was lesioned 2, but not 10 days, after learning. In addition, infusion of the glutamate antagonist CNQX, into the amygdala 5 min. before the retention test impaired inhibitory avoidance performance in rats when the retension test was given 1-5 days, but not 12 or 21 days after learning. These findings suggest that the amygdala is not the permanent memory storage site for an avoidance response. The amygdala has direct and extensive connections to all of the sensory systems in the cortex. It is likely that many of the memories are eventually stored in the cortex (Mishkin et al., 1987). The functional interchange between the sensory cortices, where memory of each sense may be stored, and the amygdala, may be critical for the ability of specific sensory input to elicit traumatic memories. Davis (1992) suggested that a highly correlated set of behaviors associated with traumatic memories may result from activation

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of the amygdala. It projects to a variety of target areas that themselves are critical for the development of these behaviors.

7. The Amygdala and its role in Stress

The amygdala is involved in the regulation of integrated behavioral, physiological, and neuroendocrine stress responses. Both peptidergic (corticotropin releasing hormone and vasopressin/oxytocin) and aminergic (noradrenaline and dopamine) systems are involved in this regulation (Bohus et al., 1996). The amygdala's modulatory role on the hypothalamo-pituitary-adrenal axis is complex. The stressors requiring interpretation with respect to previous experience ("processive stressors") reach the hypothalamic paraventricular nucleus (PVN) through the amygdala which forms intervening connections with preoptic/hypothalamic GABAergic neurons (Herman et al., 1996). Lesions placed in the CRF- and vasopressin AVP-containing neurons of the PVN increased the expression of CRF in the central amygdala and the bed nucleus of the stria terminalis, thus demonstrating that compensatory changes that take place in the amygdala could replace some of the the hypophysiotropic actions of the damaged PVN neurons (Walker et al., 1997). The amygdala's influences on adrenocorticotrophic hormone (ACTH) response to stress are either stimulatory or inhibitory, depending upon the nature of the stressor (Marcilhac and Siaud, 1996). Also, the amygdala is part of an endogenous CRF circuitry and contains a high density CRF-expressing neurons that communicate with widespread regions of the neuraxis (Gray and Bingaman, 1996). Glucocorticoid receptors are expressed in such amygdaloid CRF-containing neurons, thereby providing a contact from pituitary-adrenal activation.

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With regard to amygdaloid monoamine regulalation of stress responses, exposure to mild stressors activate dopamine (DA), serotonin (5-HT), and norepinephrine (NE) metabolism in the anteromedial prefrontal cortex (m-PFC), and the amygdala has a modulatory role in this process (Goldstein et al., 1996). In a conditioned stress model, lesions placed in the amygdala during pretraining blocked stress-induced freezing behavior, ultrasonic distress vocalizations, adrenocortical activation, and dopaminergic metabolic activation in the m-PFC. Lesions placed in the amygdala during post-training blocked stress-induced freezing and defecation, and greatly attenuated adrenocortical activation. These data provide evidence of amygdalar control of stress-induced metabolic activation of the monoaminergic systems in the m-PFC, as well as amygdalar integration of behavioral and neuroendocrine components of the rodent stress response.

8. The Amygdala and its role the processes of Pain and Analgesia

The literature on opioid manipulations in the amygdala and its putative role in the mediation of unconditioned and conditioned forms of analgesia is characterized by inconsistencies. Rodgers (1977) initially reported that the bilateral application of morphine sulfate (10 µg) into the corticomedial nuclei of amygdala resulted in hypoalgesia as indicated by an increase in the animal's threshold to respond to electric shock. However, subsequent experiments using morphine on the tail flick test (TF) failed to show any evidence of hypoalgesia (Rodgers, 1978). Yaksh and co-workers (1976) also reported that unilateral injections of morphine (5 µg) into the amygdala had little or no antinociceptive effect on the hotplate or TF test. More recent studies done by Helmstetter found that injection of morphine into the basolateral amygdaloid nucleus of pentobarbital-

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anesthetized rats dramatically elevated TF latencies (Helmstetter et al., 1993). Selective mu [D-Ala2,N-MePhe4,Gly-o]5]enkephalin, (DAMGO) or the mixed mu/kappa (MR2034) agonists in the basolateral amygdala dose-dependently elevated TF latencies, which were reversible by naltrexone(Helmstetter et al., 1995). In contrast, selective kappa (U50,488H) or delta1 ([D-Pen2,D-Pen5]enkephalin [DPDPE]) agonists failed to exert effects, suggesting that mu receptors are critical for this analgesic effect. However, little is known whether this analgesia is dependent upon either the presence of barbiturate anesthesia or possible pharmacological interactions between pentobarbitol and morphine which could be unique for this specific test preperation.

Other studies demonstrated that the full analgesic potency of systemic morphine required the central, but not the basolateral nucleus of the amygdala on the TF and formalin tests (Manning and Mayer, 1995a, 1995b).

In addition to morphine, microinjections of either neurotensin (Kalivas et al., 1982), carbachol (Klamt and Prado, 1991), the enkephalinase inhibitor SCH-32615 (Al-Rodhan et al., 1990), GABA or diazepam (Helmstetter, 1992) into the amygdala produces hypoalgesic effects on behavioral indices of nociception. Also, stimulation of the amygdala has been shown to inhibit the activity of dorsal horn spinothalamic neurons (Carstens, 1986). All studies support the suggestion that amygdala may be a component of endogenous antinociceptive circuitry. Consistent with this is the observation (Fox and Sorenson, 1994) that amygdala lesions greatly reduced analgesia induced by a non-noxious unconditioned stimulus, exposure to a cat, as well as by noxious foot-shock.

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On the other hand, some studies suggested that amygdala lesion do not affect unconditional SIA (See Pavlovic and Bodnar, 1995; Watkins et al., 1995). One of the reasons for inconsistencies of the reports about the amygdala's role in the endogenous antinociceptive circuitry comes from the use of different experimental paradigms, as well as diffferent test assays: tail-flick test in barbiturate anesthetized or awake animals (Helmstetter and Fox, 1994), jump threshold measures (Rodgers,1979), hot-plate latencies (Al-Rohdan et al., 1990) and formalin tests (Manning and Mayer, 1995), each of which may reflect differential participation of the amygdala in analgesia at different levels of the neuraxis

Multiple lines of anatomical evidence suggesting that the amygdala can receive pain information through different routes. Thousands of neurons throughout the length of the spinal cord in rats send axons directly into several hypothalamic areas (e.g. lateral hypothalamic, ventromedial, periventricular and posterior nuclei). Some of these neurones innervate other telencephalic areas including the ventral pallidum, globus pallidus, **central nucleus of the amygdala**, nucleus accumbens and infralimbic cortex, all of which are implicated in the control and expression of emotions (Giesler and Katter, 1994). It is not known however whether these projections contribute to affective changes produced by noxious stimuli. Despite the amygdala's direct anatomical connections with the hypothalamus, it is believed that the amygdala's modulation of endocrine responses to noxious somatosensory stimuli are mediated through BNST-paraventricular hypothalamic nucleus connections (Sawchenko and Swanson, 1983; Casada and Dafny, 1992).

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The amygdala also receives direct afferent projections from the dorsal raphe nucleus and the PAG (Petrov et al., 1993), which are implicated in supraspinal antinociceptive responses. One of the ways through which nociceptive information may reach the amygdala is also through an identified spino-trigemino-ponto-amygdaloid-BNST pathway, which is not very well defined (Bernard et al., 1990).

Although several different routes were described as a possible way by which the amygdala can receive pain-related information, it is stil not known whether the amygdala interacts with analgesic systems through descending projections. Some neuroanatomical, neurophysiological and pharmacological observations suggest that the amygdala induces analgesia through its connections with the PAG. The amygdala and the bed nucleus of the stria terminalis send a substantial axonal projection into the caudal ventrolateral PAG which is topographically organized in that it originates mainly from neurons in the central nucleus of the amygdala and the lateral part of the BNST (Gray et al.,1991; Holstege et al., 1985). Injections of excitatory amino acids into the ventrolateral PAG produces quiescence, hyporeactivity, hypotension, bradycardia and opioid analgesia (Bandler and Shipley, 1994). The medial subdivision of the CNA projects throughout the rostrocaudal extent of the PAG, but its density increases caudally forming dense dorsomedial and lateral-ventrolateral input columns. These columns are separated by the dorsolateral PAG which contains only moderate labeling. Neurophysiological and pharmacological evidence also suggest the presence of a functional amygdala-PAG interrelationship in the modulation of pain-inhibitory processes. Microinjections of the general opioid antagonist, naltrexone, or the mu antagonist CTOP, but not beta-endorphin 1-27, into the PAG

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significantly reduced analgesia induced by DAMGO administered into the amygdala (Tershner and Helmstetter, 1995). Therefore, an opioid synapse in the PAG is an essential link for the analgesia induced by opioid microinjections into the amygdala. Da Costa Gomez and co-workers (1995) have shown that approximately 50% of the cells in the lateral and ventrolateral columns of the PAG respond to electrophysiological (e.g. single shock or train electrical) or chemical (D,L-homosysteic acid) stimulation in the amygdala, and that this inhibitory response is mediated by a faster-conducting or more direct pathway than the pathway mediating the excitatory response. This study also found that at least one link in the CNA-PAG pathway utilizes μ or σ-opioid receptors.

These studies form the conceptual basis for the present dissertation.

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E: RATIONALE :

The amygdala is an important structure in the modulation of such homeostatic and emotional states as fear, anxiety, stress and aggression in which nociception itself or nociceptive-mediating processes can play an important role. There is emerging evidence that the amygdala plays a role in different forms of analgesic processes since certain opioid agonists can produce analgesia in particular nociceptive tests. Further, lesions placed in the amygdala disrupt the expression of certain forms of environmentally-induced analgesia, but not others. Therefore, it would be helpful to identify the neurochemical opioid receptor subtypes mediating analgesia in the amygdala, and then determine the nature of functional interactions in these pain-inhibitory processes between the amygdala and PAG. Three specific aims of this dissertation were designed to examine this issues.

1. The first specific aim of this dissertation is to examine whether analgesia can be dose-dependently elicited from the amygdala following administration of morphine and beta-endorphin. Morphine was chosen because morphine and μ-opioid agonists have been shown to elicit analgesia on the tail-flick (Helmstetter, 1992) and jump (Rodgers, 1977, 1978) tests. Beta-endorphin was chosen because it elicits powerful analgesia following ventricular administration (Jacquet and Lajtha, 1976; Bloom et al., 1976; Tseng et al., 1979) and because it sends extensive projections to the amygdala from its cell bodies in the arcuate nucleus (Watson et al., 1977, 1978; Akil et al., 1984). Further, morphine and beta-endorphin analgesia utilize different neuroanatomical and neurochemical substrates in producing their respective analgesic responses (see review: Tseng, 1995).

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These studies employed two nociceptive tests: the tail-flick test, a measure of reactivity to radiant heat (D'amour and Smith, 1941), and the jump test, a measure of reactivity to electric shock (Evans, 1961). These two measures were chosen for the following reasons. First, as noted previously, opioid-analgesia elicited from the amygdala is observed using both tests (Helmstetter, 1992; Rodgers, 1977, 1978). Second, the two tests appear to tap into nociceptive responsivity at different levels of the neuraxis. Whereas the tail-flick test is mediated at the level of the spinal cord based upon anatomical (Grossman et al., 1982) and behavioral (see reviews: Bodnar, 1986; Mayer and Manning, 1995; Terman et al., 1984) evidence, the jump test is mediated by supraspinal mechanisms. Third, while the tail-flick test is a reflexive measure that tests almost pure nociception, the jump test may be measuring other factors in addition to pain sensitivity, such as aversion. Since opioids can affect aversive responses (Bolles and Fanselow, 1980; Fanselow, 1984; Helmstetter and Fanselow, 1982; Lichtman and Fanselow, 1991), it is also possible that any increased jump thresholds following opioid microinjections into the amygdala may represent changes in aversive responsiveness as compared to nociceptive responsiveness. This would also be highly consistent with the amygdala's role in such mechanisms as fear, anxiety, and conditional emotional responses(see reviews: Davis, 1992; LeDoux, 1992).

Helmstetter and Tershner (1995) observed that an opioid synapse located in the PAG mediated μ-mediated analgesia elicited from the amygdala since general (naltrexone) and μ (CTOP) opioid antagonists, but not beta-endorphin$_{1-27}$, reduced this response. The present study will employ three opioid antagonists administered into the PAG to assess these effects upon morphine and beta-endorphin analgesia elicited from the

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amygdala. The three antagonists are naltrexone, the μ opioid antagonist, β-FNA (Portoghese et al., 1988) and the $delta_2$ opioid antagonist, NTII (Portoghese et al., 1988) Mu and $delta_2$ antagonists were chosen because μ and $delta_2$ agonists are active analgesics in the PAG (e.g. Bodnar et al., 1988; Fang et al., 1986; Jensen and Yaksh, 1984; Smith et al., 1988; Rossi et al., 1994). In contrast, neither kappa, nor $delta_1$ agonists produce analgesia in the PAG (Bodnar et al., 1988, 1991; Rossi et al., 1994). Two other important controls were used in the present study. First, it was determined whether opioid antagonists administered into the PAG alone altered baseline latencies and thresholds to investigate the possibility that any reduction in opioid analgesia from the amygdala was due to a compensatory antagonist-induced nonspecific hyperalgesia. Second, it was determined whether misplaced lateral mesencephalic placements sustained any antagonism of opioid analgesia elicited from the amygdala to investigate the anatomical specificity of the effect. Such controls were critical in determining serotonergic, opioid, NMDA and cholinergic mediation in the RVM of mesencephalic morphine analgesia (Kiefel et al., 1992a, 1992b, 1993; Spinella et al., 1996, 1997).

2. The second specific aim of this dissertation was to determine the presence of functional analgesic interactions between the amygdala and PAG for subthreshold doses of either morphine administered to each of the sites, beta-endorphin administered to each of the sites, morphine administered to one site and beta-endorphin administered to the second site, or morphine and beta-endorphin administered to each of the sites alone. Such data would indicate that these two sites interact with each other in some way as has been observed for ventricular and intrathecal opioids (Yeung and Rudy, 1980), and for

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supraspinal interactions between the PAG and RVM, PAG and LC and RVM and LC (Bodnar et al., 1991; Rossi et al., 1993, 1994). Two possibilities exist for such sites of interaction: a.) through the anatomical circuitry between the amygdala and PAG, and/or b.) through mechanisms initiated from these two sites converging at yet another site or sites. Such interactions were evaluated using the tail-flick and jump tests.

3. The third and the last specific aim of this dissertation was to examine whether the amygdala is a supraspinal site of analgesic action for kappa-mediated agonists. Although kappa-mediated agonists elicit analgesia following ventricular and intrathecal administration, $kappa_1$ selective drugs, like U50488H fail to produce analgesia following microinjections into the PAG, RVM or LC (Bodnar et al., 1991, Jensen and Yaksh, 1986; Rossi et al., 1994). If ethylketocyclazocine is administered simultaneously to the PAG and LC, it produces a μ_1-mediated analgesia that is blocked by naloxonazine (Bodnar et al., 1991). However, U50488H fails to produce such an interaction. Helmstetter and co-workers (1995) failed to find analgesic activity of U50488H in the amygdala on the tail-flick test in barbiturate-anesthetized rats. The present study evaluated the analgesic activity of the $kappa_1$ agonist, U50488H in the amygdala on the tail-flick and jump tests in awake, behaving rats, assessed its kappa selectivity in the amygdala by pretreatment with the $kappa_1$ antagonist, nor-binaltorphamine (Nor-BNI: Portoghese et al., 1987) and examined whether PAG opioid synapses participate in the mediation of these responses with general (naltrexone), μ (β-FNA) and $delta_2$ (NTII) antagonists.

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II. 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 h light: 12 h dark cycle with food and water available ad libitum. Following anesthetization with chlorpromazine HCl (3 mg/kg, ip) and Ketamine HCl (120 mg/kg, im), two stainless steel guide cannulae (26 gauge, Plastics One, Roanoke, VA) were placed stereotaxically (Kopf Instruments) into the amygdala and either the PAG (Experiments 1, 2 and 3) or a control misplaced mesencephalic site lateral to the PAG (Experiment 1). Stereotaxic coordinates were: (a) amygdala: incisor bar (-3.3 mm), 2.8 mm posterior to the bregma suture, 5 mm lateral to the sagittal suture and 8.2 mm from the top of the skull (Experiments 1, 2 and 3), (b) PAG incisor bar (-5 mm), 0.3-0.6 mm anterior to the lambda suture, 1.5-2.0 mm lateral to and angled 12° toward the sagittal suture, and 6.5-7.0 mm from the top of the skull (Experiments 1, 2 and 3), and misplaced control: incisor bar (-5 mm), 0.3-0.6 mm anterior to the lambda suture, 2.0 mm lateral to the sagittal suture, and 6.5-7.0 mm from the top of the skull (Experiment 1). Cannulae were secured to 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, H. 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 um sections through both cannulae placements. The tissue, stained with Cresyl violet were examined by light microscopy by

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an observer unfamiliar with the behavioral data. Only animals with confirmed cannula placements were included in the data analysis.

<u>**Nociceptive tests:**</u> In a given session, each animal was tested on the tail-flick <u>and</u> jump tests in that order to minimize carry-over effects between tests in all experiments. A tail-flick analgesiometer (IITC Co.) produced a radiant heat source that was mounted 8 cm above a photocell upon which the rat's tail was placed. Radiant heat was applied 3-9 cm proximal to the tip of the rat's tail; removal of the tail activated the photocell and determined the latency (0.01 sec accuracy). The thermal intensity of the radiant heat source 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 intertrial intervals. To avoid tissue damage, a trial was automatically terminated if a response did not occur within 12 s. Rats were tested on the jump test immediately after tail-flick testing. Electric shock was delivered to the feet of the rat by a shock generator (BRS/LVE) and 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 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 the jump threshold was determined. A cut-off threshold of 1.2 mA was used for all jump threshold trials. Baseline latencies and thresholds were determined for at least 4 days before experimental testing began to insure stability of responding. All animals displayed consistent latencies and thresholds in baseline and vehicle testing that did not appear subject to desensitization.

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Drugs: Morphine (Pennick Laboratories, Experiments 1 and 2), beta-endorphin (Peninsula Laboratories, Experiments 1 and 2), U50488H (Upjohn Pharmaceuticals, Experiment 3), naltrexone (Sigma Chemical Co., Experiments 1 and 3), Nor-BNI (Research Biochemicals, Experiment 3) and BFNA (Research Biochemicals, Experiments 1 and 3) were dissolved in normal saline. Ntii (Research Biochemicals, Experiments 1 and 3) was initially dissolved in DMSO at a concentration of 10 mg/ml, and then diluted to the test dose with normal saline. All microinfusions were administered at 4-8 h into the light cycle in 1 ul volumes at a rate of 0.2 ul every 10 s through a stainless steel internal cannula (33 gauge, Plastics One) which was connected to a Hamilton microsyringe by polyethylene tubing. Respective mesencephalic microinjections of either naltrexone (20 min), BFNA (24 h) or Ntii (24 h) preceded amygdala morphine, beta-endorphin or U50488H 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). Amygdala microinjections of NorBNI preceded amygdala U50488H by 30 min. to reflect peak antagonist activity.

Statistical analyses: Repeated measures analyses of variance were performed to assess significant effects among experimental conditions and across the time course with Dunnett comparisons ($p<.05$) determining the potency of either morphine, beta-endorphin or U50488H analgesia relative to vehicle/vehicle treatment. Dunn comparisons ($p<.05$) determined antagonist effects relative to corresponding vehicle/agonist treatment for Experiments 1 and 3. Peak analgesia was derived by subtracting the vehicle/vehicle score at 90 min from each respective and corresponding experimental score. Total analgesia was

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derived by subtracting the sum of the four vehicle/ vehicle scores (30-120 min) from the sum of each of the respective experimental scores (30-120 minutes).

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III. EXPERIMENT I

INTRODUCTION:

Although evaluation of anatomical substrates mediating supraspinal opioid analgesia has mostly centered upon brainstem structures, including the PAG, LC and RVM (Basbaum and Fields, 1984; Bodnar et al., 1988, 1991; Fields and Basbaum, 1978; Fields et al., 1991), the amygdala has also been implicated in analgesic processes. Increased nociceptive thresholds are observed following microinjections of either morphine (Helmstetter and Tershner, 1993; Rodgers, 1977, 1978), an enkephalinase inhibitor (Al-Rodhan and Yaksh, 1990), neurotensin (Kalivas et al., 1982) or carbachol (Klamt and Prado, 1991) into the central and baso-lateral amygdaloid nuclei.

The amygdala, particularly the central nucleus, has high concentrations of enkephalins (Finley and Petrusz, 1981; Hokfelt et al., 1977; Khachaturian et al., 1982) relative to dynorphins (Khachaturian et al., 1982) and has high concentrations of opiate receptors in binding (see review: Fowler and Fraser, 1994), autoradiographic (Mansour et al., 1987) and mRNA in situ hybridization (Mansour and Watson, 1995) studies. Amygdala nuclei capable of eliciting analgesic responses have both intrinsic and extrinsic connections with mesencephalic pain-modulatory nuclei, especially the PAG and dorsal raphe nucleus (Beitz, 1982; deOlmos, Alheid and Beltramino, 1985; Krettek and Price, 1978; Nitecka et al., 1981; Rizvi et al., 1991). The first aim of the present study was to evaluate analgesic effectiveness of two opioid agonists in the amygdala: morphine and beta-endorphin.

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Differences between morphine and beta-endorphin analgesia occur at forebrain and the PAG levels. Although analgesia induced by either morphine or beta-endorphin in the PAG was blocked by either general or mu-selective antagonists (e.g., CTOP: Smith and Monroe, 1992), beta-endorphin analgesia is differentially sensitive to these antagonists when compared with morphine (Hawranko et al., 1995; Monroe and Smith, 1996), suggesting the involvement of separate receptors. The present study investigated the existence of a functional relationship between the amygdala and PAG in analgesic processes by determining whether opioid analgesia induced by either morphine or beta-endorphin from the amygdala could be altered by pretreatment with opioid antagonists in the PAG. Since opioid modulation of analgesia in the PAG acts through mu and $delta_2$ receptors, the present study examined whether opioid analgesia elicited from the amygdala was altered by pretreatment with either naltrexone (general), BFNA (mu: Portoghese and Takemori, 1990; Takemori and Portoghese, 1981) or Ntii ($delta_2$: Mattia et al., 1992; Portoghese and Takemori, 1990) as measured by the tail-flick and jump tests. To establish specificity of these effects, the ability of opioid antagonists in the PAG to alter basal nociceptive latencies and thresholds was investigated, as well as the ability of opioid antagonists administered in lateral mesencephalic control misplacements to alter opioid agonist-induced analgesia from the amygdala. This experiment was published in Brain Research (741: 13-26, 1996).

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PROCEDURES

Five different protocols were employed as summarized in Table I: a) opioid antagonists in the PAG and morphine in the amygdala, b) opioid antagonists in misplaced mesencephalic placements and morphine in the amygdala, c) opioid antagonists in the PAG and beta-endorphin in the amygdala, d) opioid antagonists in misplaced mesencephalic placements and beta-endorphin in the amygdala, and e) opioid antagonists in the PAG. In Protocols A-D, tail-flick latencies and jump thresholds were determined 30, 60, 90 and 120 min following the second microinjection of each pair. In Protocol E, tail-flick latencies and jump thresholds were determined at 30-min intervals at either 0.5 or 24 h following vehicle, at 0.5 h following naltrexone, and at 24 h following either BFNA or Ntii. In each protocol, all animals received the vehicle treatment, and in Protocols A-D, all animals treated with opioid antagonists received the particular dose of agonist used in subsequent antagonist tests. Animals receiving particular antagonist doses were matched on the basis of their basal nociceptive latencies and thresholds as well as the magnitude of either morphine or beta-endorphin analgesia at a 5 ug dose in the amygdala. There was a one week interval between particular treatment conditions; this interval minimized agonist-induced tolerance effects and long-term antagonist effects in previous studies (Bodnar et al., 1988, 1991; Kiefel and Bodnar, 1992, 1993, Smith et al., 1992). No animal in any protocol received more than four microinjection conditions in a given site.

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TABLE I. Summary of experimental groups.

Mesencephalic Placements	Amygdala Placement	n
A.Opioid Antagonists (PAG) - Morphine (Amygdala)		
Vehicle	Vehicle	19
Vehicle	Morphine (2.5 ug)	12
Vehicle	Morphine (5 ug)	19
Naltrexone (1ug)	Morphine (5 ug)	14
Naltrexone (5 ug)	Morphine (5 ug)	15
BFNA (4 ug)	Morphine (5 ug)	8
Ntii (4 ug)	Morphine (5 ug)	8
B.Opioid Antagonists (Misplaced) - Morphine (Amygdala)		
Vehicle	Vehicle	5
Vehicle	Morphine (5 ug)	5
Naltrexone (5 ug)	Morphine (5 ug)	5
BFNA (4 ug)	Morphine (5 ug)	5
Ntii (4 ug)	Morphine (5 ug)	5
C.Opioid Antagonists (PAG) - Beta-Endorphin (Amygdala)		
Vehicle	Vehicle	11
Vehicle	BE (1 ug)	5
Vehicle	BE (5 ug)	11
Naltrexone (1 ug)	BE (5 ug)	5
Naltrexone (5 ug)	BE (5 ug)	5
BFNA (4 ug)	BE (5 ug)	6
Ntii (4 ug)	BE (5 ug)	6
D.Opioid Antagonists (Misplaced) - Beta-Endorphin (Amygdala)		
Vehicle	Vehicle	7
Vehicle	BE (5 ug)	7
Naltrexone (5 ug)	BE (5 ug)	6
BFNA (4 ug)	BE (5 ug)	7
Ntii (4 ug)	BE (5 ug)	6
E.Opioid Antagonists (PAG)		
Vehicle	—	7
Naltrexone (5 ug)	—	6
BFNA (4 ug)	—	7
Ntii (4 ug)	—	7

Note: BFNA: beta-funaltrexamine; Ntii: Naltrindole isothiocyanate; BE: beta-endorphin; PAG: Periaqueductal gray; Misplaced: Sites lateral and ventral to the PAG.

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RESULTS

Histological verification: Figure 1 summarizes the histological verification of cannula placements in the amygdala which were located in the medial (n=6), basolateral (n=10), basomedial (n=9), central (n=7) and posterior basolateral (n=10) nuclei. Figures 1A and 1B display representative cannula placements in the basolateral and central amygdala nuclei respectively. Rats in the morphine and beta-endorphin treatment protocols had rostral cannula placements in the above nuclei of the amygdala, and evaluation of the overall magnitude of morphine and beta-endorphin analgesia revealed no significant differences between these nuclei on either the tail-flick or jump tests. Therefore, data for each agonist treatment for the two amygdala placements were pooled for antagonist analysis. There were no systematic differences in cannula placements in the amygdala as functions of the morphine and beta-endorphin treatment groups and of the appropriate and misplaced mesencephalic groups. Finally, placements (n=4) dorsal and medial to the amygdala failed to alter tail-flick latencies and jump thresholds following morphine and beta-endorphin (data not shown).

Figure 2 summarizes the schematic histological verification of cannula placements in the PAG which were located in the lateral and ventrolateral PAG from the level of the III cranial nerve to the dorsal raphe. Control mesencephalic placements were located in the far lateral mesencephalic reticular formation.

Amygdala Morphine Analgesia: Significant differences in latencies and thresholds were observed among microinjection conditions (tail-flick: $F(6,126)= 5.58$, $p<.0001$; jump: F 32.45, $p<.0001$), across test times (tail-flick: $F(3,378)= 5.88$,

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FIGURE 1 Cannula placements in the amygdala as represented using the atlas of Paxinas and Watson (1968). Cannula placements were located in the basolateral (upper panel) and central (lower panel) nuclei of the amygdala.

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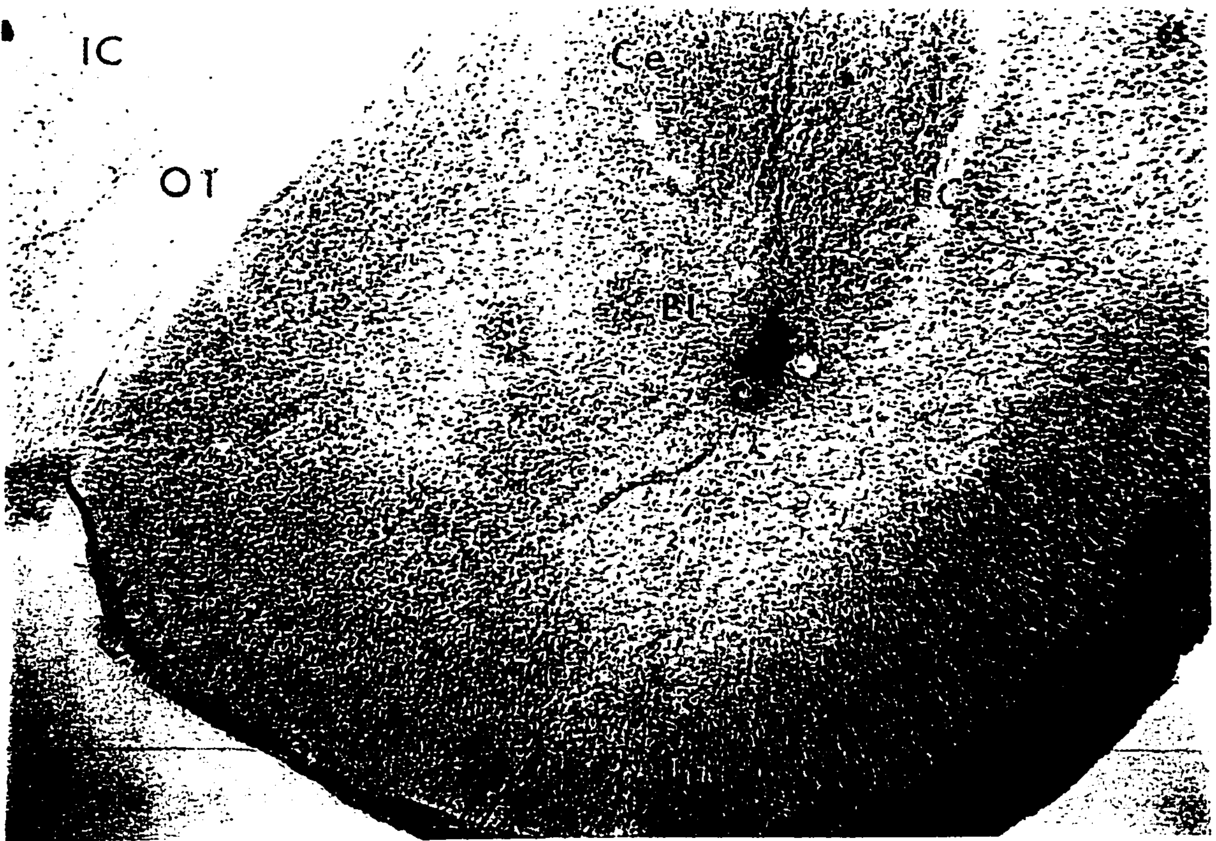

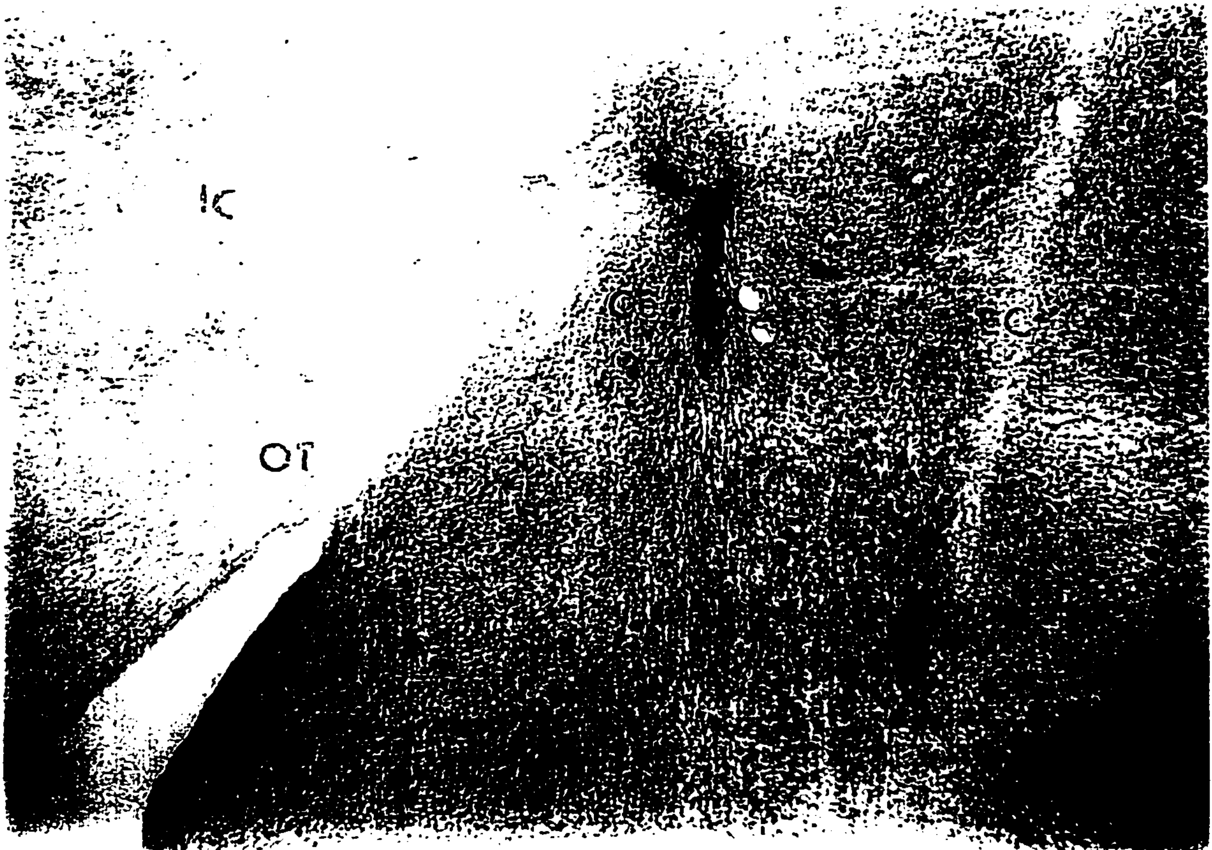

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FIGURE 2. PAG cannulae were located in the lateral PAG at the level of the III cranial nerve (f: n=9), the ventral PAG at the level of the III cranial nerve (g: n=11), the ventrolateral PAG (h: n=8), the dorsal raphe nucleus (i: n=4), and the lateral PAG at the level of the dorsal raphe nucleus (l: n=5). Control mesencephalic placements were located in the far lateral mesencephalic reticular formation at (j: n=6) and slightly ventral (k: n=6) to the dorsal-ventral extent of the cerebral aqueduct.

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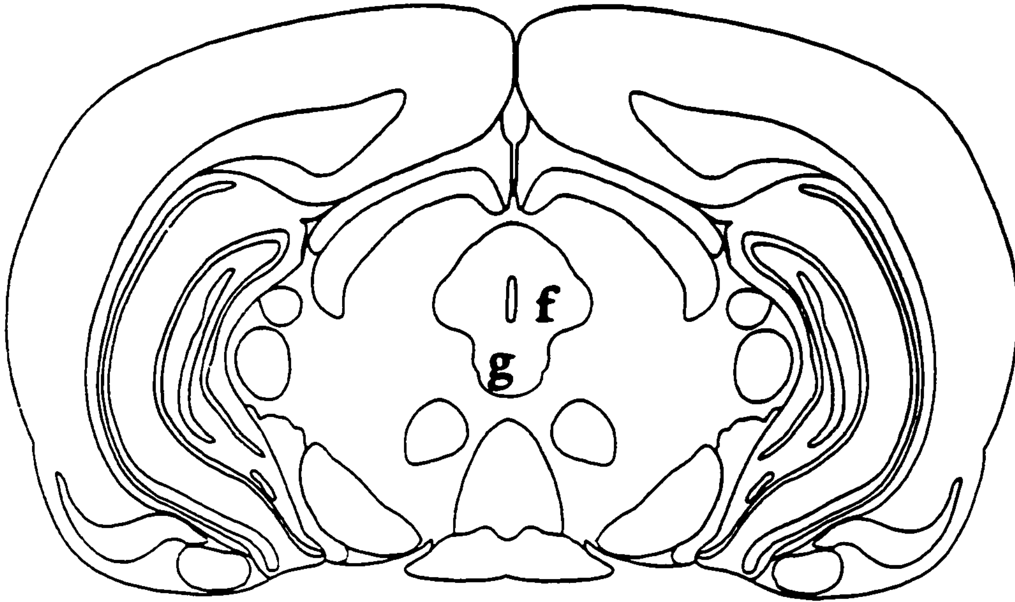

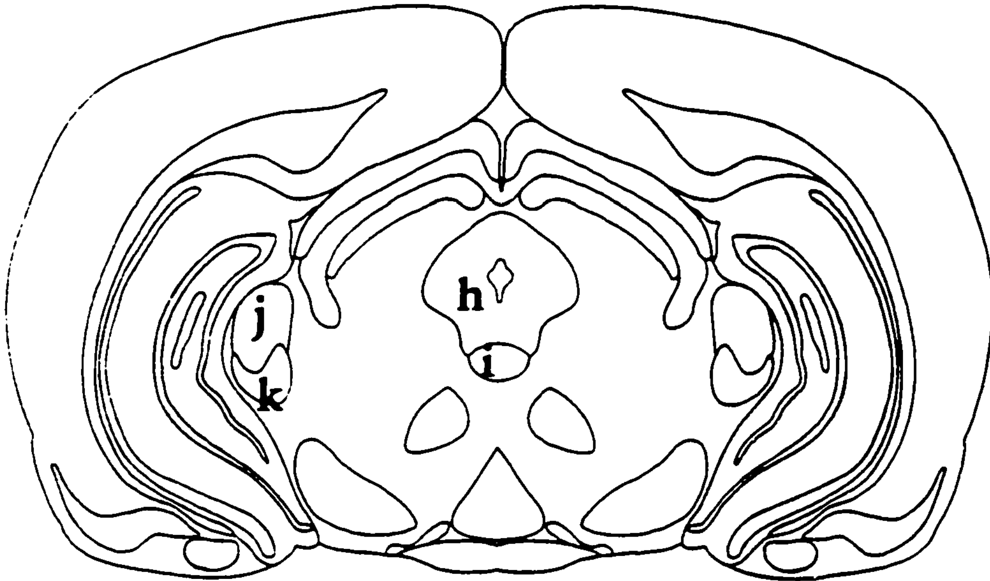

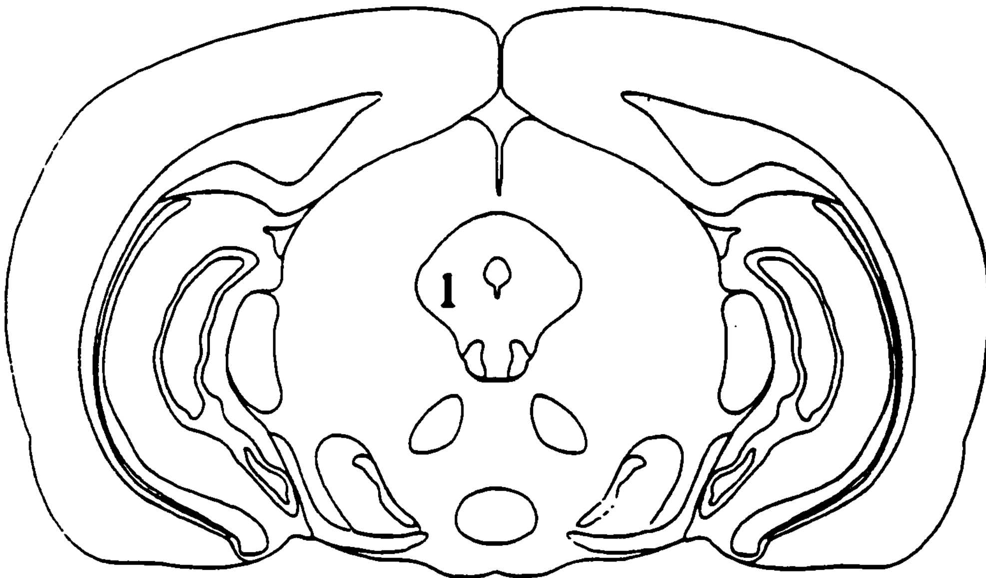

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$p<.0006$; jump: $F= 22.66$, $p<.0001$) and for the interaction between conditions and times (tail-flick: $F(18,378)= 2.55$, $p<.0005$; jump: $F= 10.65$, $p<.0001$). Morphine microinjections in the amygdala significantly increased tail-flick latencies following the 2.5 (30, 90 min) and 5 (30-120 min) ug doses (Figure 3, upper panel). However, the magnitude of increase was small, reflecting a 1.0-1.6 sec increase in latencies. Morphine microinjections in the amygdala significantly increased jump thresholds following the 2.5 (60-120 min) and 5 (30-120 min) ug doses (Figure 3, lower panel). These effects were more pronounced, reflecting nearly a doubling of baseline thresholds. Peak analgesic responses for both measures occurred 90 min following morphine microinjection.

PAG Opioid Antagonist and Amygdala Morphine Analgesia: Naltrexone microinjections in the PAG significantly reduced morphine analgesia elicited from the amygdala on the tail-flick (1 ug: 30-90 min; 5 ug: 30-120 min) and jump (1 ug: 60-120 min; 5 ug: 30-120 min) tests (Figure 4A and 4B, respectively). Significant decreases were observed for both peak (tail-flick: 69-71%; jump: 60-81%) and total (tail-flick: 70-75%; jump: 44-79%) effects. Administration of the $delta_2$ opioid antagonist, Ntii in the PAG significantly reduced morphine analgesia elicited from the amygdala on both tests across the time course, eliminating peak and total effects on the tail-flick test, and reducing peak (53%) and total (60%) effects on the jump test (Figure 5C and 5D, respectively). In contrast, administration of the mu opioid antagonist, BFNA in the PAG significantly reduced morphine analgesia elicited from the amygdala only after 90 min. on the tail-flick test, and only after 30 and 90 min. on the jump test (Figure 5C and 5D). In fact, PAG BFNA significantly increased amygdala morphine analgesia on the jump test after 120 min.

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FIGURE 3. Alterations in tail-flick latencies (left panel) and jump thresholds (right panel) in rats microinjected with morphine (M: 0, 2.5, 5 ug) in the amygdala. Significant increases in latencies and thresholds following morphine microinjection relative to vehicle treatment are denoted by crosses (Dunnett comparisons, $p<.05$).

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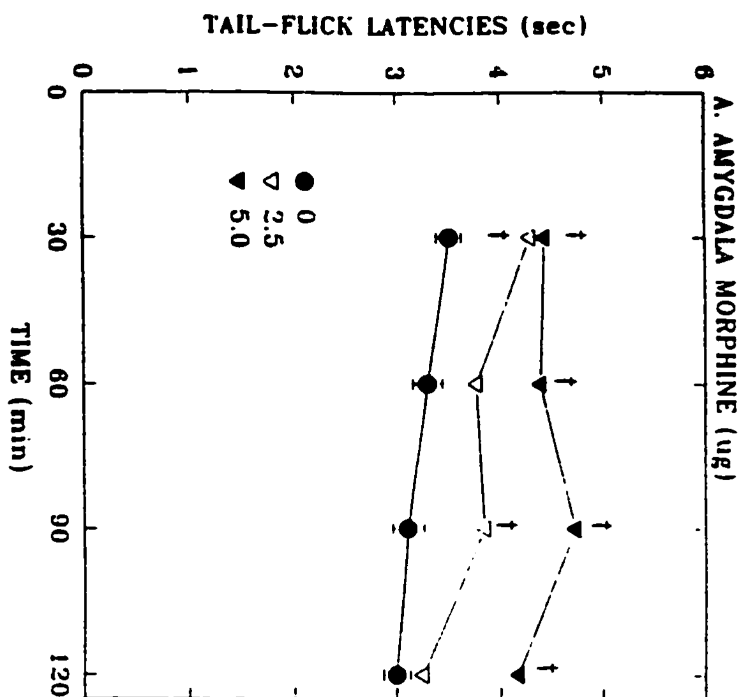

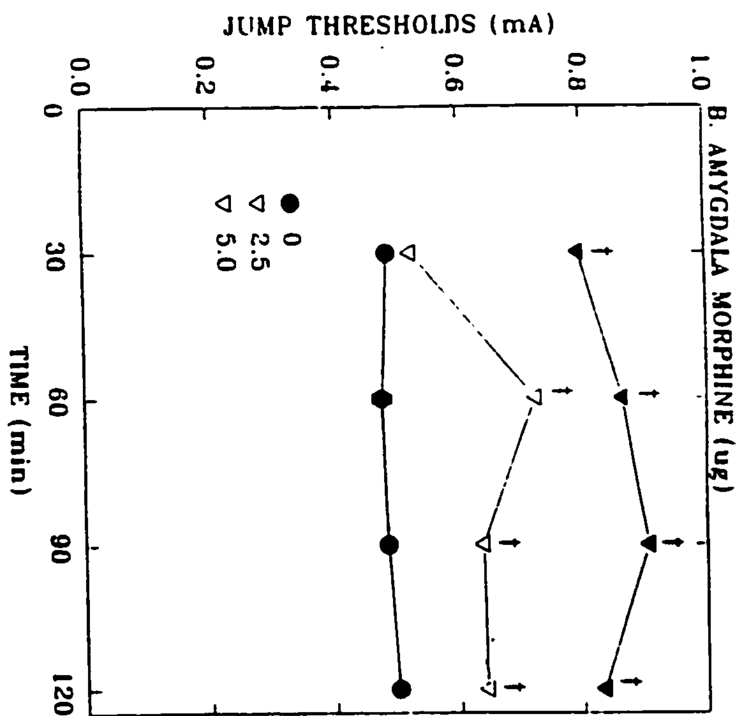

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FIGURE 4. Alterations in tail-flick latencies (Panel A) and thresholds (Panel B) in rats microinjected with morphine (M) in the amygdala 20 min following pretreatment with naltrexone (N: 0, 1 or 5 ug). Significant alterations in morphine analgesia by opioid antagonists are denoted by stars (Dunn comparisons, $p<.05$), and significant differences in morphine effects relative to vehicle treatment are denoted by crosses (Dunnett comparisons, $p<.05$).

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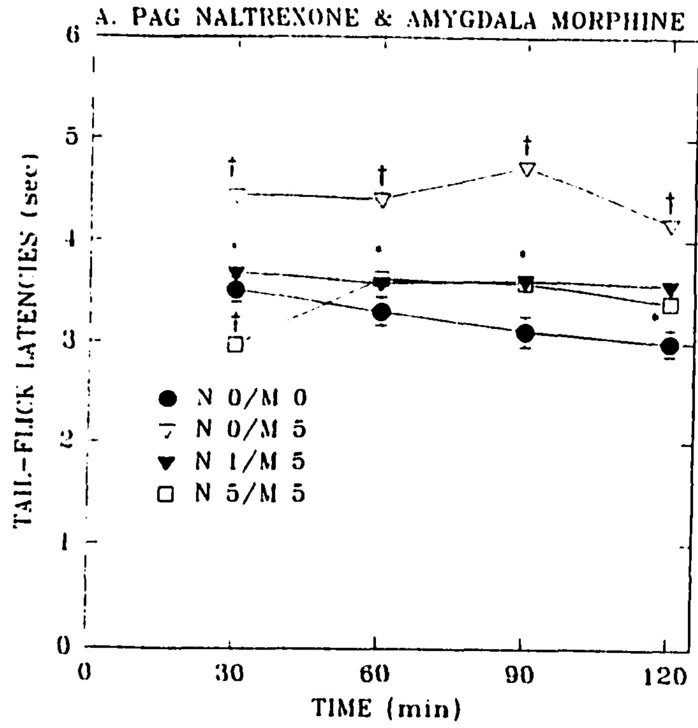

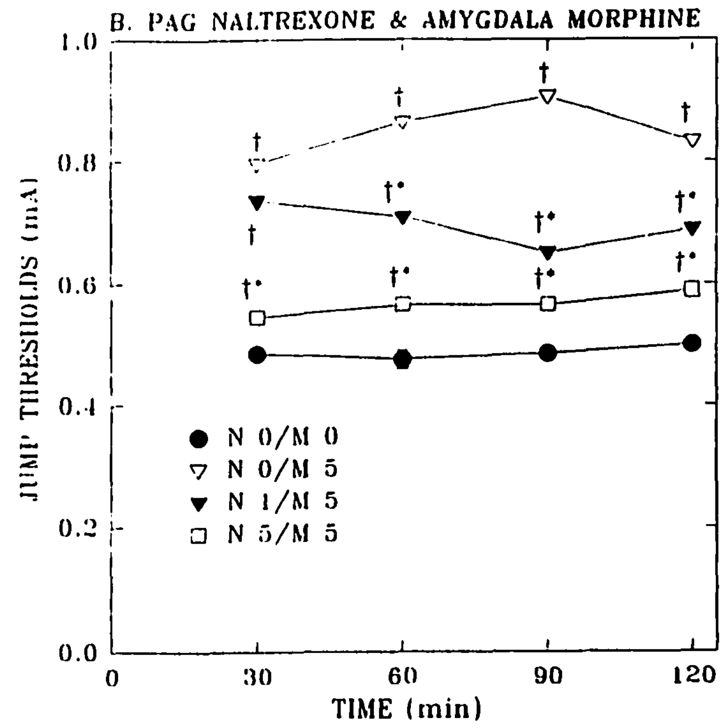

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FIGURE 5. Alterations in tail-flick latencies (Panel C) and thresholds (Panel D) in rats following 24 h pretreatment with beta-funaltrexamine (B-FNA: 4 ug) or naltrindole isothiocyanate (Ntii: 4 ug) microinjected into the PAG prior to morphine (M) in the amygdala. Significant alterations in morphine analgesia by opioid antagonists are denoted by stars (Dunn comparisons, $p<.05$), and significant differences in morphine effects relative to vehicle treatment are denoted by crosses (Dunnett comparisons, $p<.05$).

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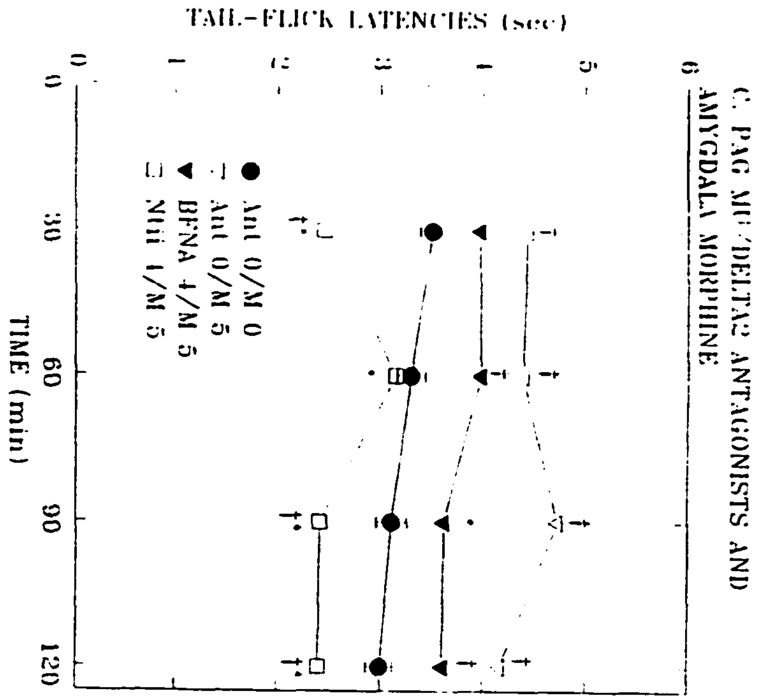

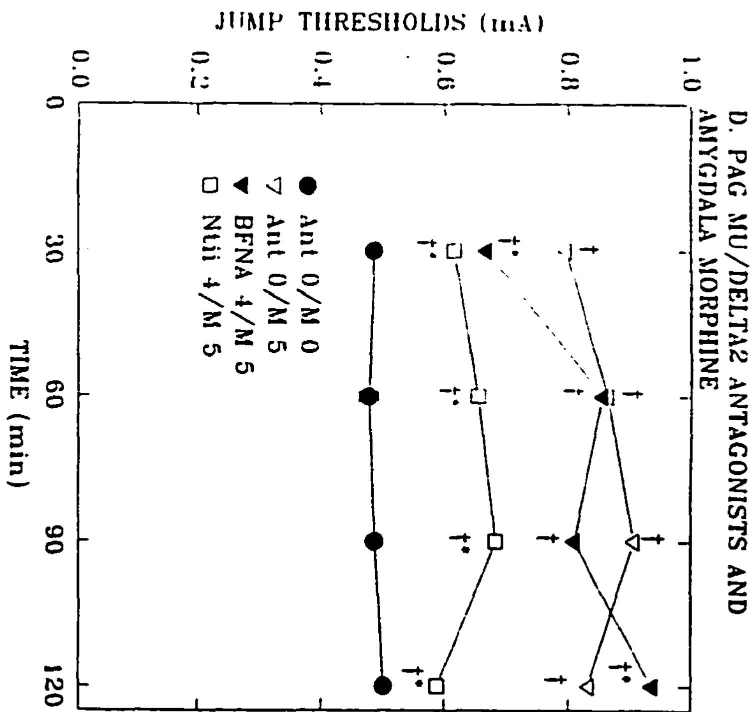

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While PAG BFNA reduced peak (68%) and total (53%) analgesic effects on the tail-flick test, it had no appreciable effects on peak (24%) and total (11%) analgesic responses on the jump test.

The specificity of opioid antagonist effects in the PAG upon morphine analgesia elicited from the the amygdala was tested using groups of rats with control misplaced mesencephalic placements. Significant differences in latencies and thresholds were observed among conditions (tail-flick: $F(6,90) = 4.27$, $p < .009$; jump: $F = 8.44$, $p < .0002$), across test times (tail-flick: $F(3,75) = 0.65$, ns; jump: $F = 18.20$, $p < .0001$) and for the interaction between conditions and times (tail-flick: $F(12,75) = 0.70$, ns; jump: $F = 3.91$, $p < .0001$). Both naltrexone and BFNA microinjections in the lateral mesencephalic control placements actually significantly increased morphine analgesia elicited from the amygdala across the time course on the tail-flick test (Table II). Further, morphine analgesia elicited from the amygdala on the jump test was significantly increased following lateral mesencephalic control microinjections of naltrexone (30 min), BFNA (30, 90 min) and Ntii (90 min) (Table II).

Amygdala Beta-Endorphin Analgesia: Significant differences in latencies and thresholds were observed among microinjection conditions (tail-flick: $F(6,77) = 37.74$, $p < .0001$; jump: $F = 16.19$, $p < .0001$), across test times (tail-flick: $F(3, 231) = 3.76$, $p \angle .012$; jump: $F = 44.41$, $p \angle .0001$) and for the interaction between conditions and times (tail-flick: $F(18,231) = 3.61$, $p < .0001$; jump: $F = 7.76$, $p < .0001$). Beta-endorphin microinjections in the amygdala significantly increased tail-flick latencies at 60, 90 and 120 min following the 1 ug dose and across the time course following the 5 ug dose (Figure 6, left panel). The

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TABLE II. Alterations in either morphine or beta-endorphin analgesia elicited from the amygdala by pretreatment with either naltrexone, BFNA or Ntii in mesencephalic sites lateral to the PAG.

Condition	Post-injection time (min)			
	30	60	90	120
A. Morphine Analgesia (Tail-Flick Latencies (s)):				
Veh/Veh	2.75	2.70	2.93	3.20
Veh/Mor	2.57	2.67	2.58	2.53
Ntx/Mor	4.84*+	4.47*+	4.79*+	4.56*+
BFNA/Mor	4.44*+	4.30*+	4.52*+	4.08*+
Ntii/Mor	2.97	2.60	2.54	2.37#
B. Morphine Analgesia (Jump Thresholds (mA)):				
Veh/Veh	.535	.521	.557	.537
Veh/Mor	.645*	.788*	.712*	.707*
Ntx/Mor	.747*+	.819*	.782*	.605*
BFNA/Mor	.742*+	.807*	.868*+	.671*
Ntii/Mor	.691*	.753*	.892*+	.705*
C. Beta-Endorphin Analgesia (Tail-Flick Latencies (s)):				
Veh/Veh	2.54	2.32	2.42	2.26
Veh/BE	3.04	2.50	2.65	2.19
Ntx/BE	2.90	3.37*+	3.03	3.02
BFNA/BE	2.94	2.66	2.58	2.75*+
Ntii/BE	3.38*+	3.01*+	2.53	2.72
D. Beta-Endorphin Analgesia (Jump Thresholds (mA)):				
Veh/Veh	.580	.588	.588	.567
Veh/BE	.731*	.791*	.758*	.696*
Ntx/BE	.899*+	.856*+	.807*	.721*
BFNA/BE	.781*+	.811*	.812*+	.761*+
Ntii/BE	.790*+	.860*+	.778*	.742*

Note: Ntx: Naltrexone; BFNA: beta-funaltrexamine; Ntii: Naltrindole isothiocyanate BE: beta-endorphin; Mor: Morphine; PAG: Periaqueductal gray. *Significantly increase from Veh/Veh; +Significant increase relative to Veh/Mor or Veh/BE; #Significant decrease relative to Veh/Veh.

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FIGURE 6. Alterations in latencies (left panel) and thresholds (right panel) in rats microinjected with beta-endorphin (BE: 0, 2.5, 5 ug) in the amygdala. Significant increases in latencies and thresholds following beta-endorphin microinjection relative to vehicle treatment are denoted by crosses (Dunnett comparisons, $p<.05$).

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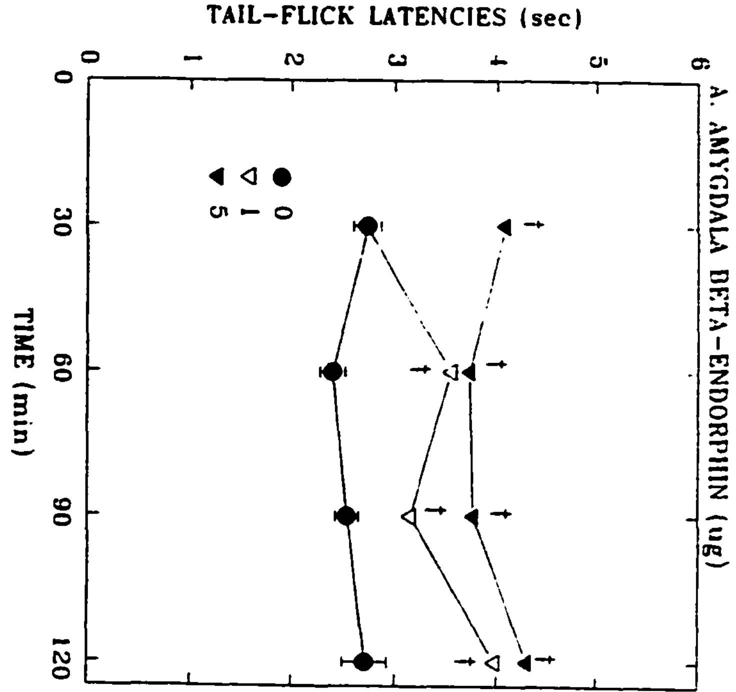

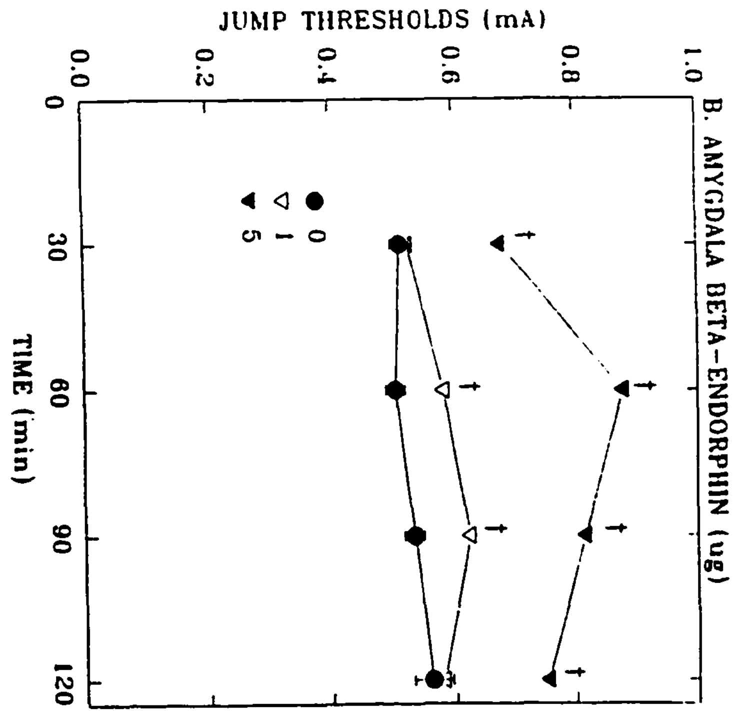

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magnitude of these significant increases were again small, reflecting a 1.0-1.6 sec increase in latencies.

B-endorphin microinjections in the amygdala significantly increased jump thresholds at 60 and 90 min following the 1ug dose and across the time course following the 5ug dose (Figure 6, right panel). This analgesic magnitude was again more pronounced on the jump test, reflecting nearly a doubling of baseline thresholds. Peak analgesic responses for both measures occurred 60 min following beta-endorphin microinjection.

PAG Opioid Antagonists and Amygdala Beta-Endorphin Analgesia: PAG naltrexone dose-dependently altered beta-endorphin analgesia elicited from the amygdala on the tail-flick (1 ug: 30-120 min; 5 ug: 30-120 min) and jump (1 ug: 30 and 90 min; 5 ug: 30-120 min) tests (Figure 7A and 7B, respectively). The lower dose of naltrexone in the PAG significantly reduced beta-endorphin analgesia elicited from the amygdala on the tail-flick test, but increased analgesia on the jump test. The higher naltrexone dose in the PAG significantly reduced beta-endorphin analgesia elicited from the amygdala for both the tail-flick (peak and total: 100%) and jump (peak: 86%; total: 93%) tests. Ntii in the PAG significantly reduced beta-endorphin analgesia elicited from the amygdala on the jump test for both peak and total effects (79%) (Figure 8D), but actually increased analgesic effects on the tail flick test 930 and 90 min: 21-24%; Figure 8C, left panel). BFNA in the PAG failed to significantly alter beta-endorphin analgesia elicited from the amygdala for either peak or total effects on either test. The specificity of opioid antagonist

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FIGURE 7. Alterations in latencies (Panel A) and thresholds (Panel B) in rats microinjected with beta-endorphin (BE) in the amygdala 20 min following pretreatment with naltrexone (N: 1 or 5 ug). Significant alterations in beta-endorphin analgesia by opioid antagonists are denoted by stars (Dunn comparisons, $p < .05$), and significant differences in beta-endorphin effects relative to vehicle treatment are denoted by crosses (Dunnett comparisons, $p < .05$).

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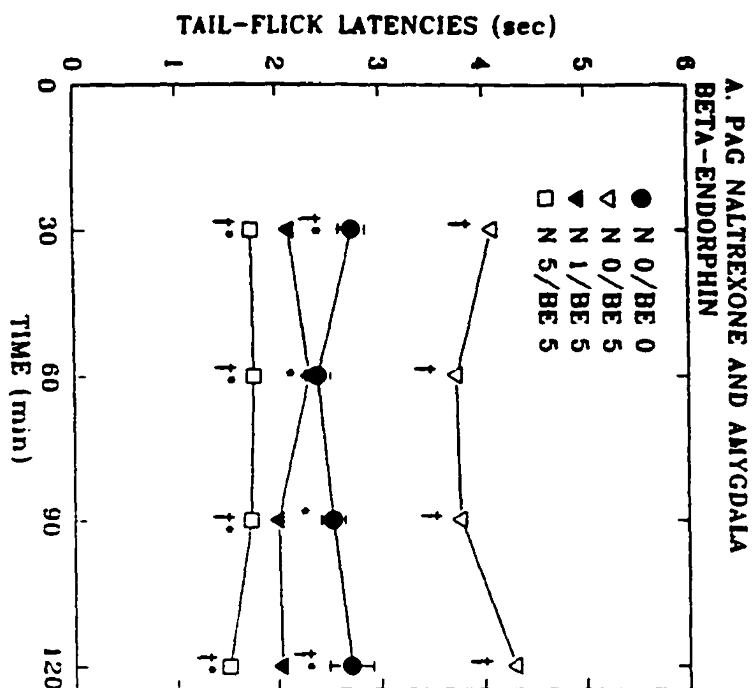

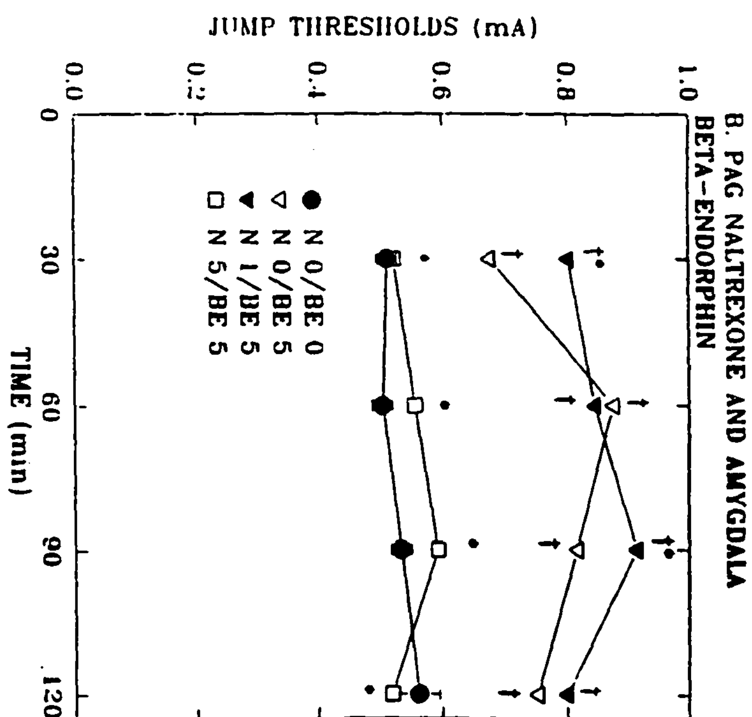

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FIGURE 8. Alterations in latencies (Panel C) and thresholds (Panel D) in rats microinjected with beta-endorphin (BE) in the amygdala, 24 h following pretreatment with either B-FNA (4 ug) or Ntii (4 ug) microinjected into the PAG. Significant alterations in beta-endorphin analgesia by opioid antagonists are denoted by stars (Dunn comparisons, $p<.05$), and significant differences in beta-endorphin effects relative to vehicle treatment are denoted by crosses (Dunnett comparisons, $p<.05$).

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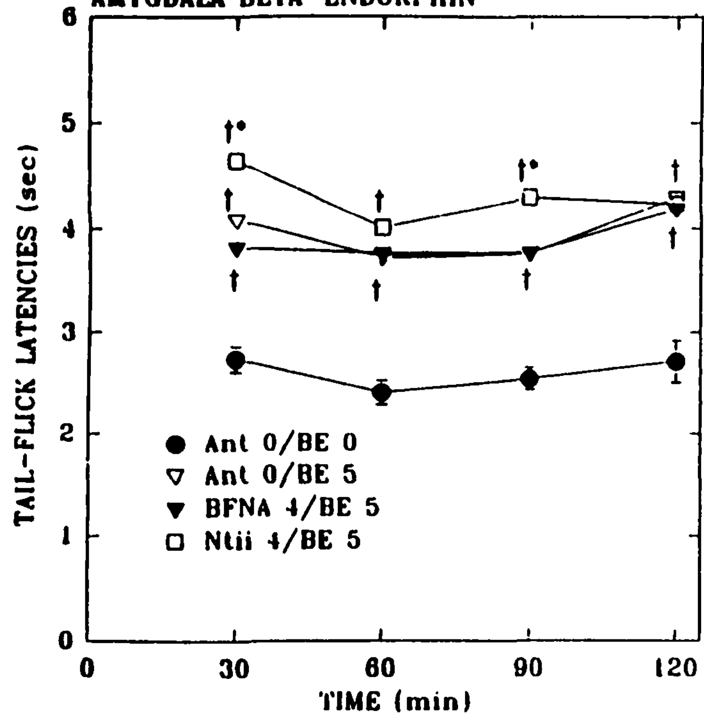

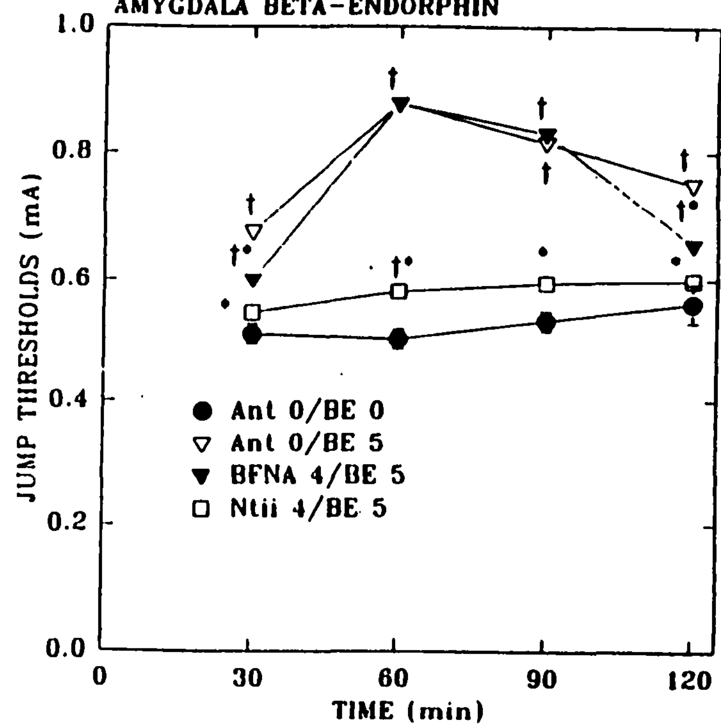

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effects in the PAG upon beta-endorphin analgesia elicited from the the amygdala was also tested using control placements.

Significant differences in latencies and thresholds were observed among conditions (tail-flick: $F(4,120)= 0.94$, ns; jump: $F= 14.80$, $p<.0001$), across test times (tail-flick: $F(3,105)= 4.13$, $p<.008$; jump: $F= 23.11$, $p<.0001$) and for the interaction between conditions and times (tail-flick: $F(12,75)= 1.64$, ns; jump: $F= 4.12$, $p<.0001$). Beta-endorphin analgesia elicited from the amygdala on the tail-flick test test was significantly increased following lateral mesencephalic control microinjections of naltrexone (60, 120 min) and BFNA (120 min) (Table II). Further, beta-endorphin analgesia elicited from the amygdala on the jump test was significantly increased following lateral mesencephalic control microinjections of naltrexone (30-60 min), BFNA (30, 90, 120 min) and Ntii (30-60 min) (Table II).

Opioid Antagonist Effects in the PAG: In assessing whether opioid antagonists possessed intrinsic effects in the PAG upon nociceptive thresholds themselves, significant differences failed to be observed among conditions (tail-flick: $F(3,84)= 2.01$, ns; jump: $F= 0.60$, ns), but were observed across test times (tail-flick: $F(3,72)= 9.75$, $p<.0001$; jump: $F= 2.71$, $p<.05$) and for the interaction between conditions and times (tail-flick: $F(9,72)= 0.96$, ns; jump: $F= 2.25$, $p<.028$). Tail-flick latencies were unaffected following antagonist treatments in the PAG (Table III). In contrast, jump thresholds were significantly reduced following PAG microinjections of either naltrexone (5 ug: 90-120 min), BFNA (4 ug: 90-120 min) or Ntii (4 ug: 90 min). However, the magnitude of hyperalgesic effects induced by naltrexone (9-12%), BFNA (6-7%) and Ntii (9%) in the PAG did not approach the

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TABLE III. Alterations in tail-flick latencies and jump thresholds following opioid antagonists in the PAG.

Condition	Post-injection time (min)			
	30	60	90	120
A. Tail-Flick Latencies (s):				
Veh	2.70	2.49	2.39	2.61
Ntx	2.71	2.49	2.40	2.48
BFNA	3.02	2.65	2.55	2.44
Ntii	2.83	2.30	2.06	2.43
B. Jump Thresholds (mA):				
Veh	.526	.507	.549	.518
Ntx	.500	.478	.483*	.469*
BFNA	.528	.530	.513*	.482*
Ntii	.514	.499	.500*	.519

Note: Ntx: naltrexone; BFNA: beta-funaltrexamine; Ntii: Naltrindole isothiocyanate PAG: Periaqueductal gray. *Significantly decrease from Veh.

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magnitude of antagonist-induced reductions of either morphine or beta-endorphin analgesia elicited from the amygdala.

DISCUSSION

Morphine and Beta-Endorphin Analgesia in the Amygdala: The present study demonstrated that morphine and beta-endorphin microinjected into the amygdala significantly increased tail-flick latencies and jump thresholds in rats. Morphine analgesia elicited from the amygdala confirms previous reports of this effect on both the tail-flick and jump tests (Helmstetter and Tershner, 1993; Rodgers, 1977, 1978). The analgesia elicited by beta-endorphin in the amygdala is consistent with observed beta-endorphin projections from the hypothalamic arcuate nucleus to the amygdala (Bloom et al., 1978; Watson et al., 1977, 1978). The magnitude of amygdala morphine and beta-endorphin analgesia was far greater on the jump test than on the tail-flick test, though both tests were assessed in the same animals over the same time course in an order in which the first test fails to alter the threshold of the second test (Kelly, 1982). Typically, morphine and beta-endorphin in the amygdala increased tail-flick latencies by less than 2 sec, and produced these effects at doses (2.5-5 ug) that would produce maximal analgesic responses in the PAG and LC, and sizable responses in the RVM (Bodnar et al., 1988; Fang et al., 1986; Jacquet and Lajtha, 1973; Smith et al., 1988; Yaksh and Rudy, 1976).

The test-specific differences in the potencies of morphine and beta-endorphin analgesia elicited from the amygdala may reflect the relative participation of the amygdala in differentially mediating each pain test at different levels of the neuraxis. Whereas the tail-flick test (D'Amour and Smith, 1941) is mediated at the level of the spinal cord based

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upon anatomical (Grossman, Basbaum and Fields, 1982) and behavioral (see reviews: Bodnar, 1986; Terman et al., 1984) evidence, the jump test is mediated by supraspinal mechanisms. The greater sensitivity of shock-reactive stimuli as compared to reflexive thermal stimuli to analgesic processes in the amygdala is consistent with this nucleus' role in such mechanisms as fear, anxiety and conditioned emotional responses (see reviews: Davis, 1992, LeDoux, 1992). Further, morphine and beta-endorphin in the amygdala produced larger analgesic responses on the jump test in which the greatest magnitude of effects occurred at 60-90 min following microinjection rather than at shorter (e.g., 30 min) intervals. These data are consistent with the time course of DAMGO analgesia in the amygdala which displays a gradual increase in magnitude over a 20 min time course (Helmstetter et al., 1995). The amygdala nuclei responsible for these analgesic responses are under question. Helmstetter and co-workers (Helmstetter et al., 1993) indicated that the baso-lateral nucleus supported higher magnitudes of DAMGO and morphine analgesia than the central nucleus. In contrast, Manning and Mayer (1995) found that lesions placed in the central, but not the baso-lateral amygdala nuclei produce rightward shifts in the potency of systemic morphine analgesia. The present study found analgesia could be elicited from both sites with both agonists, but not from placements medial or dorsal to theamygdala. However, the injection volume (1 ul) used in the present study makes it difficult to precisely determine the spread of agonists from the cannula tips in both nuclei.

PAG Naltrexone and Amygdala Opioid Analgesia: The present study indicated that an opioid synapse in the PAG was integral for the full expression of morphine and beta-endorphin analgesia elicited from the amygdala. Naltrexone (1-5 ug) in

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the PAG significantly and substantially reduced morphine and beta-endorphin analgesia elicited from the amygdala on the tail-flick and jump tests. These effects occurred for opioid agonist-induced analgesia from either baso-lateral or central amygdala nuclei. The specificity of these effects were confirmed by the inability of naltrexone administered into misplaced control sites in the lateral mesencephalon to alter opioid agonist-induced analgesia in the amygdala. Further, the small and sporadic hyperalgesia elicited by naltrexone in the PAG could not account for its substantial effects on opioid agonist-induced analgesia in the amygdala. These data agree with a recent observation (Tershner and Helmstetter, 1995) that PAG naltrexone significantly reduced DAMGO analgesia elicited from the baso-lateral amygdala in barbiturate-anesthetized rats. This effect was observed if naltrexone was administered in the PAG prior to, but not following DAMGO administration in the amygdala. Thus, PAG naltrexone can block, but not reverse DAMGO analgesia. Together, these data provide behavioral and functional significance in analgesic processes for the anatomical connections between the amygdala and PAG (Beitz, 1982; deOlmos et al., 1985; Krettek and Price,1978; Nitecka et al., 1981; Rizvi et al., 1991). They also complement a recent neurophysiological study (Da Costa Gomez and Behbehani, 1995) demonstrating that electrical or chemical stimulation of the central nucleus of the amygdala produced excitatory or inhibitory effects in approximately 50% of identified PAG cells. The inhibitory response in the PAG was mediated by a faster-conducting or more direct pathway than the excitatory response, and naloxone was more effective in blocking the inhibitory response than the excitatory response.

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The present evidence cannot specify whether the opioid synpase in the PAG mediating opioid agonist analgesia elicited from the amygdala is either an enkephalinergic pathway projecting from the amygdala to the PAG (Cassel et al., 1986; Hokfelt et al., 1977; Moss et al., 1983), or PAG interneurons containing opioid peptides (see review: Akil et al., 1984). However, these data add to the growing list of interconnected nuclei in which either analgesic synergy can be observed following subthreshold microinjections of opioid agonists (e.g., PAG, RVM, LC: Rossi and Bodnar, 1993, 1994), or an opioid antagonist applied to one site can block opioid analgesia elicited from a second site. Thus, general opioid antagonists administered into either the RVM (Kiefel and Bodnar, 1993) or habenula (Ma and Han, 1992) blocks morphine analgesia elicited from the PAG, and naloxone administered into the nucleus accumbens blocks morphine analgesia elicited from the habenula (Ma and Han, 1992).

PAG Opioid Receptor Subtypes and Amygdala Opioid Analgesia: The present study also investigated which opioid receptor subtypes in the PAG (mu, delta, kappa: see reviews: Mansour and Watson, 1987,1995) modulated morphine and beta-endorphin analgesia elicited from the amygdala. While mu and $delta_2$ receptors appear to mediate PAG morphine analgesia in agonist, antagonist and synergy studies (Bodnar et al., 1988, 1991, Fang et al., 1986; Jensen and Yaksh, 1986; Kiefel et al., 1993, Rossi et al., 1993, Smith et al., 1988, 1992), $delta_1$ and kappa receptor agonists are ineffective in the PAG (Bodnar et al., 1988, 1991; Rossi et al., 1994; Smith et al., 1988). PAG administration of the $delta_2$ opioid antagonist, Ntii produced greater reductions in both morphine and beta-endorphin analgesia elicited from the amygdala on the jump test than PAG administration

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of the mu antagonist, BFNA. Previous evidence indicates that these antagonists act upon distinct receptor subtypes since analgesia elicited by the mu-selective agonist, DAMGO in the PAG is blocked by PAG BFNA, but not naltrindole, while analgesia elicited by the $delta_2$-selective agonist, deltorphin II in the PAG is blocked by PAG naltrindole, but not BFNA (Rossi et al., 1994). $Delta_2$ and mu antagonist effects upon morphine and beta-endorphin analgesia in the amygdala appeared specific to the PAG since misplaced lateral mesencephalic microinjections could not produce these effects, and since the antagonists alone in the PAG produced only small, sporadic decreases in nociceptive thresholds. The question of test specificity was also raised using selective antagonists. While PAG BFNA and Ntii blocked the small increases in tail-flick latencies elicited by morphine in the amygdala, neither PAG antagonist altered the small increases in tail-flick latencies elicited by beta-endorphin in the amygdala. Tershner and Helmstetter (1995) have provided complementary data regarding opioid receptor subtype mediation in the PAG of amygdala opioid analgesia. DAMGO analgesia in the amygdala of barbiturate-anesthetized rats was blocked by the short-acting mu antagonist, CTOP on the tail-flick test, parallelling our PAG BFNA effects upon amygdala morphine analgesia on this measure. In contrast, administration of the putative epsilon receptor antagonist, beta-$endorphin_{1-27}$ in the PAG failed to alter DAMGO analgesia elicited from the amygdala. These data agree with previous observations that morphine and beta-endorphin analgesia are dissociated from each other when the tail-flick test is utilized (Monroe et al., 1996; Smith et al., 1992; Suh and Tseng, 1988, 1989, 1990, 1992;Tseng and Collins, 1991, 1992; Tseng and Suh, 1989; Tseng and Tang, 1990). That beta-endorphin and morphine analgesia elicited from the

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amygdala may be mediated by multiple ($delta_2$ and mu) opioid receptor subtypes in the PAG is not surprising given the differential sensitivity of beta-endorphin and morphine analgesia elicited from the PAG to different opioid antagonists (Monroe et al., 1996; Smith et al., 1992). Indeed, these antagonist studies in the PAG reveal the possibility of both pain-inhibitory and pain-facilitatory actions of beta-endorphin in mediating nociceptive responses and stress-induced analgesia (Hawranko et al., 1994, 1995). Thus, it appears that mu and $delta_2$ receptors comprise the opioid synapse in the PAG mediating different forms of opioid analgesia elicited from the amygdala, and indicates a site of action whereby forebrain mechanisms involved in analgesic processes connect with the intrinsic brainstem and spinal pathways critically involved in pain control.

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IV. EXPERIMENT II

INTRODUCTION:

One of the means of evaluating functional connections between brain structures in mediating analgesic responses is to determine the presence of interactions. Synergistic analgesic interactions have been observed between supraspinal (intracerebroventricular, icv) and spinal (intrathecal, it) sites for subthreshold doses of morphine (Miyamoto et al., 1991; Pick and Pasternak, 1992, 1993; Roerig et al., 1984; Siuciak and Advocat, 1989; Yeung and Rudy, 1980), beta-endorphin (Roerig and Tseng, 1988) and selective opioid agonists (Roerig and Fujimoto, 1989). Given the fact that functional connections between the amygdala and PAG have been observed in the mediation of analgesic responses, the aim of the present study was to determine the presence of functional analgesic interactions between the amygdala and PAG for subthreshold doses of either morphine administered to each of the sites, beta-endorphin administered to each of the sites, or morphine administered to one site and beta-endorphin administered to the second site. There are two possibilities for the site(s) of interaction: a) through the anatomical circuitry between the amygdala and PAG, and/or b) through mechanisms initiated from these two sites converging at yet another site or sites.

Both the tail-flick test, a nociceptive measure of thermal reactivity (D'Amour and Smith, 1941) and the jump test, a measure of shock reactivity (Evans, 1961) were employed because the magnitude of analgesic responsivity in the amygdala appears to be more potent for the jump test relative to the tail-flick test (Helmstetter et al., 1993, 1995; Pavlovic and Bodnar, 1996; Rodgers, 1977, 1978; Tershner and Helmstetter, 1995;

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Yaksh, Yeung and Rudy, 1976). Comparisons of interactions between subthreshold doses of morphine and beta-endorphin in the amygdala and PAG were done because each of these opioid agonists appear to utilize different neurochemical substrates in mediating their respective analgesic responses (see review: Tseng, 1995).

This experiment has been accepted for publication in the journal, Brain Research.

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PROCEDURES

Single-injection protocols: Each rat received a maximum of four single microinjection conditions at weekly intervals. Analgesic dose-response curves were assessed for morphine (1-5 ug) and beta-endorphin (1-5 ug) on the tail-flick and jump tests at 30, 60, 90 and 120 min in the amygdala alone and in the PAG alone. Table IV summarizes the doses and sample sizes for each condition.

Double-injection protocols: Each rat received a maximum of four double microinjection conditions at weekly intervals. In the first phase of the double injection protocol, morphine-morphine interactions were assessed between the amygdala and PAG on the tail-flick and jump tests after 30, 60, 90 and 120 min. Thus, morphine at a fixed subthreshold dose (1 ug) was administered into one site (e.g., the amygdala) and morphine at varied subthreshold doses (0.05-1 ug) was administered into the second site (e.g., the PAG). Then the second site received a fixed subthreshold dose of morphine, and the first site received varied subthreshold doses of morphine. In the second phase of the double injection protocol, beta-endorphin-beta-endorphin interactions were assessed between the amygdala and PAG on the tail-flick and jump tests after 30, 60, 90 and 120 min. Again, the rationale of fixed and varied doses between sites was applied as in the first phase, except that beta-endorphin was applied to both sites. In the third phase of the double injection protocol, interactions between morphine applied to one site and beta-endorphin applied to the second site (and vice-versa) were assessed between the amygdala and PAG on the tail-flick and jump tests after 30, 60, 90 and 120 min. In the fourth and final phase

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TABLE IV. Alterations (±SEM) in tail-flick latencies following single or paired administration (ug) of either morphine (MOR) or beta-endorphin (BE) into the amygdala and periaqueductal gray (PAG).

Amygdala Injections	PAG Injections	n	Peak Effects	Total Effects
A. Single Morphine Injection:				
0	0	19	2.72 (0.10)	10.84 (0.38)
1		8	3.07 (0.09)	12.26 (0.23)
2		5	2.67 (0.10)	11.46 (0.31)
5		6	3.10 (0.18)	12.07 (0.43)
	1	8	3.15 (0.12)*	12.23 (0.51)*
	2	5	4.37 (0.24)*	18.46 (1.15)*
	5	5	8.41 (0.29)*	29.70 (0.85)*
B. Paired Morphine Injections:				
0.05	1	7	4.94 (0.64)*+#	15.72 (1.48)*+#
0.25	1	8	3.11 (0.24)	12.50 (0.60)
1	0.05	6	2.51 (0.10)	9.85 (0.28)
1	0.25	7	2.81 (0.14)	11.30 (0.48)
1	1	7	2.98 (0.16)	12.34 (0.54)
C. Single Beta-Endorphin Injection:				
0	0	23	2.53 (0.07)	10.31 (0.31)
1		8	2.17 (0.05)	9.43 (0.38)
2		6	2.24 (0.05)	8.73 (0.20)
5		6	4.11 (0.26)*	14.99 (0.51)*
	1	9	2.57 (0.14)	10.18 (0.35)
	2	7	2.30 (0.03)	10.97 (0.61)
	5	6	6.86 (0.50)*	26.45 (0.73)*
D. Paired Beta-Endorphin Injections:				
0.05	1	6	2.87 (0.19)*+#	11.88 (0.56)*+#
0.25	1	7	3.06 (0.20)*+#	12.01 (0.48)*+#
1	0.05	6	2.88 (0.20)*	10.78 (0.40)
1	0.25	7	2.81 (0.05)*	11.42 (0.38)*
1	1	9	2.56 (0.09)	11.15 (0.30)*+#
E. Mixed Morphine/Beta-Endorphin Injections:				
1 (BE)	1(MOR)	8	3.79 (0.94)*	12.04 (1.99)
1 (MOR)	1(BE)	8	2.13 (0.14)	8.97 (0.45)
1 (BE)/ 1(MOR)		7	2.57 (0.15)	11.04
	1 (BE)/1 (MOR)	8	3.79 (0.94)*	13.95 (1.97)

*Significant differences relative to vehicle (*) or to a 1 (+) or 2 (#) ug dose of MOR or BE (Tukey comparisons, $p<.05$).

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of the double injection protocol, interactions between morphine and beta-endorphin administered simultaneously to the same site were assessed for the amygdala and PAG on the tail-flick and jump tests after 30, 60, 90 and 120 min. Table IV also summarizes the doses and sample sizes for each of these four series of conditions.

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RESULTS

Histological Verification: The 59 rats in the morphine and beta-endorphin treatment protocols had rostral cannula placements in the central (n=27) and baso-lateral (n=32) nuclei of the amygdala. The caudal cannula placements were located in the lateral (n=9), ventrolateral (n=13) and ventral (n=8) PAG at the level of the III cranial nerve, the dorsal raphe nucleus (n=8), and the lateral (n=9) and ventrolateral (n=12) PAG at the level of the dorsal raphe nucleus. These placements are highly similar to those described and depicted previously (Pavlovic and Bodnar, 1996). Evaluation of the overall magnitude and pattern of morphine and beta-endorphin analgesia revealed no significant differences between these nuclei on both nociceptive tests. Further, there were no systematic differences in cannula placements as functions of the different treatment paradigms.

Morphine Analgesia in the Amygdala and Interactions with the PAG: Significant differences were observed among experimental conditions (tail-flick: $F(6,105)= 10.37$, $p<.0001$; jump: $F= 19.32$, $p<.0001$), across test times (tail-flick: $F(3,315)= 23.63$, $p<.0001$; jump: $F= 22.70$, $p<.0001$) and for the interaction between conditions and times (tail-flick: $F(18,315)= 6.33$, $p<.0001$; jump: $F= 9.14$, $p<.0001$). Whereas morphine produced marginal and transitory increases in tail-flick latencies following microinjection into the amygdala (Table IV), morphine dose-dependently increased jump thresholds following the 2 and 5 ug, but not the 1 ug doses in the amygdala (Figure 9A). When morphine doses of either 0.05, 0.25 or 1 ug in the amygdala were paired with a fixed (1 ug), ineffective (see next section) morphine dose in the PAG, jump thresholds were significantly increased across the time course relative to either the 0, 1 or 2 ug morphine

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doses in the amygdala alone (Figure 9B). In contrast, morphine interactions between the amygdala and PAG were not observed on the tail-flick test, except for small, but significant increases in latencies following simultaneous administration of 1 ug of morphine into the PAG and 0.05 ug of morphine into the amygdala (Table IV).

Morphine Analgesia in the PAG and Interactions with the Amygdala: Significant differences were observed among experimental conditions (tail-flick: $F(6,84)=$ 22.09, $p<.0001$; jump: $F= 20.12$, $p<.0001$), across test times (tail-flick: $F(3,252)= 23.87$, $p<.0001$; jump: $F= 15.97$, $p<.0001$) and for the interaction between conditions and times (tail-flick: $F(18,252)= 6.19$, $p<.0001$; jump: $F= 7.15$, $p<.0001$). Morphine dose-dependently increased tail-flick latencies (Table IV) and jump thresholds (Figure 10C) with potent effects following the 2 and 5 ug doses, and marginal effects following the 1 ug dose in the PAG. When morphine doses of either 0.05, 0.25 or 1 ug in the PAG were paired with a fixed (1 ug), ineffective (see previous section) morphine dose in the amygdala, jump thresholds were significantly increased across the time course relative to either the 0 or 1 ug morphine doses in the PAG alone (Figure 10D). Pairing morphine microinjections of 1 ug into the amygdala and 1 ug into the PAG also significantly increased jump thresholds relative to a morphine doses of 2 ug in the PAG alone (Figure 10D). In contrast, morphine interactions between the amygdala and PAG failed to occur on the tail-flick test (Table IV).

Beta-Endorphin Analgesia in the Amygdala and Interactions with the PAG: Significant differences were observed among experimental conditions (tail-flick: $F(6,126)=$ 22.41, $p<.0001$; jump: $F= 55.94$, $p<.0001$), across test times (tail-flick: $F(3,378)= 37.70$,

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FIGURE 9. Alterations (Mean, ±SEM) in jump thresholds in rats receiving morphine (M) in the amygdala alone (Panel A), or a fixed dose of morphine (1 ug) in the periaqueductal gray (PAG) simultaneously paired with morphine (M: 0.05-1 ug) into the amygdala (Panel B).

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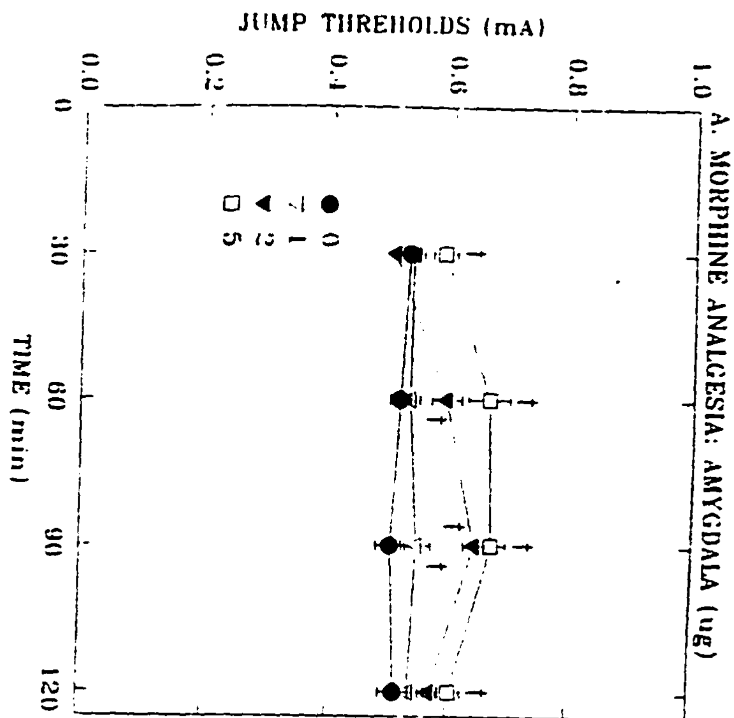

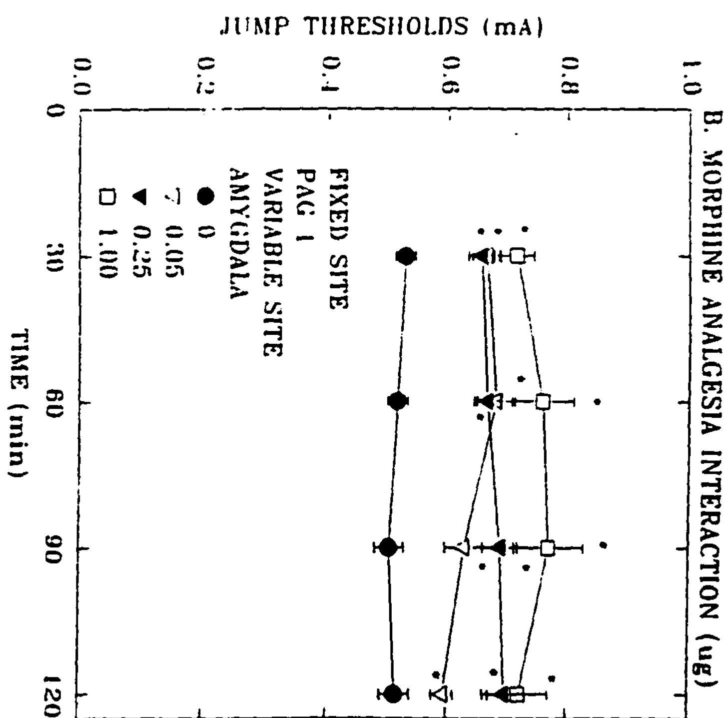

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FIGURE 10. Alterations (Mean, ±SEM) in jump thresholds in rats receiving morphine into the PAG alone (Panel C), or a fixed dose of morphine (1 ug) in the amygdala simultaneously paired with morphine (0.05-1 ug) in the PAG (Panel D). Tukey comparisons ($p<.05$) revealed significant differences in jump thresholds for morphine relative either to vehicle treatment (crosses), or to morphine at either a 1 ug (#) or a 2 (*) ug dose into a single site.

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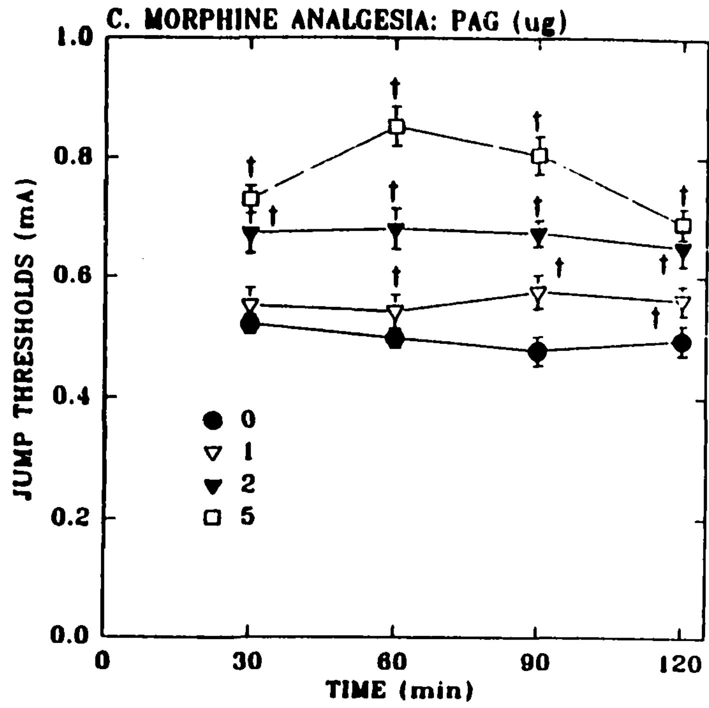

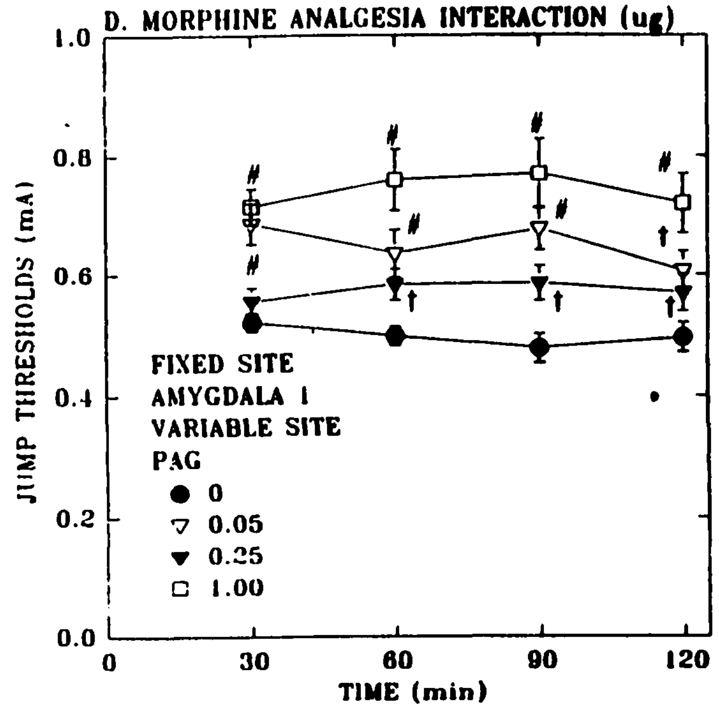

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p<.0001; jump: F= 118.55, p<.0001) and for the interaction between conditions and times (tail-flick: F(18,378)= 8.78, p<.0001; jump: F= 18.00, p<.0001). Whereas beta-endorphin failed to increase tail-flick latencies following microinjection into the amygdala (Table IV), beta-endorphin dose-dependently increased jump thresholds across the time course following the 2 and 5 ug, but not the 1 ug doses in the amygdala (Figure 11A). When beta-endorphin doses of either 0.05, 0.25 or 1 ug in the amygdala were paired with a fixed (1 ug), ineffective (see next section) beta-endorphin dose in the PAG, jump thresholds were significantly increased across the time course relative to either the 0, 1 or 2 ug beta-endorphin doses in the amygdala alone (Figure 11B). Beta-endorphin interactions between the amygdala and PAG occurred on the tail-flick test, but only increased latencies by less than 1 sec (Table IV).

Beta-Endorphin Analgesia in the PAG and Interactions with the Amygdala: Significant differences were observed among experimental conditions (tail-flick: F(6,147)= 123.60, p<.0001; jump: F= 85.65, p<.0001), across test times (tail-flick: F(3,441)= 154.29, p<.0001; jump: F= 61.14, p<.0001) and for the interaction between conditions and times (tail-flick: F(18,441)= 85.93, p<.0001; jump: F= 18.11, p<.0001). Beta-endorphin dose-dependently increased tail-flick latencies (Table IV) and jump thresholds (Figure 12C) following the 2 and 5 ug, but not the 1 ug doses in the PAG. When beta-endorphin doses of either 0.05, 0.25 or 1ug in the PAG were paired with a fixed (1ug), ineffective (see previous section) beta-endorphin dose in the amygdala, jump thresholds were significantly increased across the time course relative to either the 0, 1 or 2 ug beta

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FIGURE 11. Alterations (Mean, ±SEM) in jump thresholds in rats receiving beta-endorphin in the amygdala alone (Figure 11: Panel A), or a fixed dose of beta-endorphin (BE: 1 ug) in the PAG simultaneously paired with beta-endorphin (BE: 0.05-1 ug) in the amygdala (Figure 11: Panel B)

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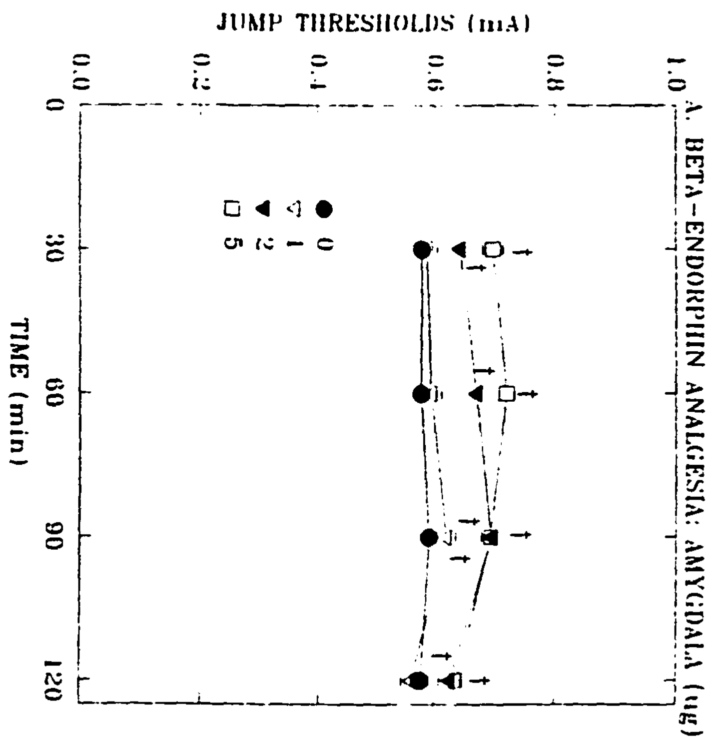

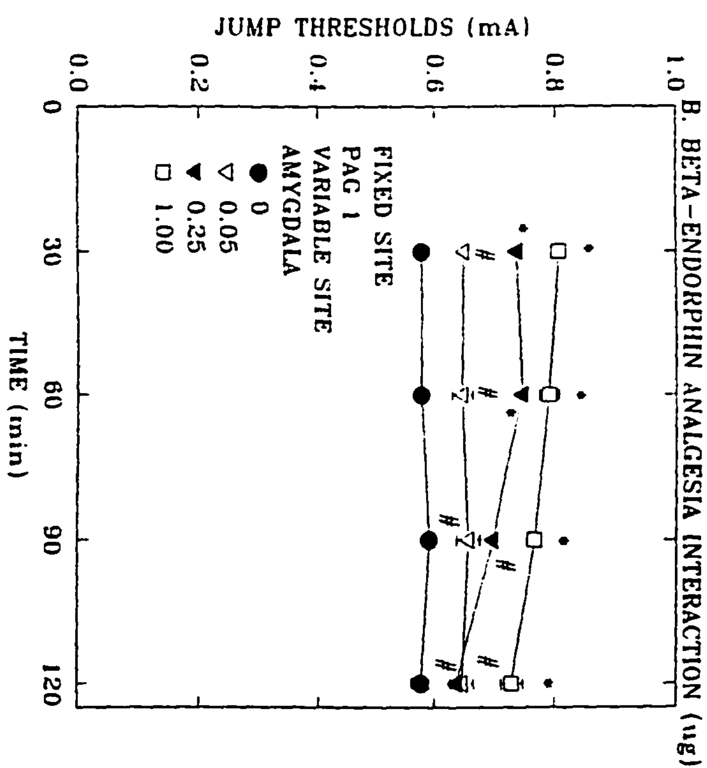

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FIGURE 12. Alterations (Mean, ±SEM) in jump thresholds in rats receiving beta-endorphin in the PAG alone (Figure 12: Panel C), or a fixed dose of beta-endorphin (BE: 1 ug) in the amygdala simultaneously paired with beta-endorphin (BE: 0.05-1 ug) in the PAG (Figure 12: Panel D). Tukey comparisons ($p<.05$) revealed significant differences in jump thresholds for beta-endorphin relative either to vehicle treatment (crosses), or to beta-endorphin at either a 1 ug (#) or a 2 (*) ug dose into a single site.

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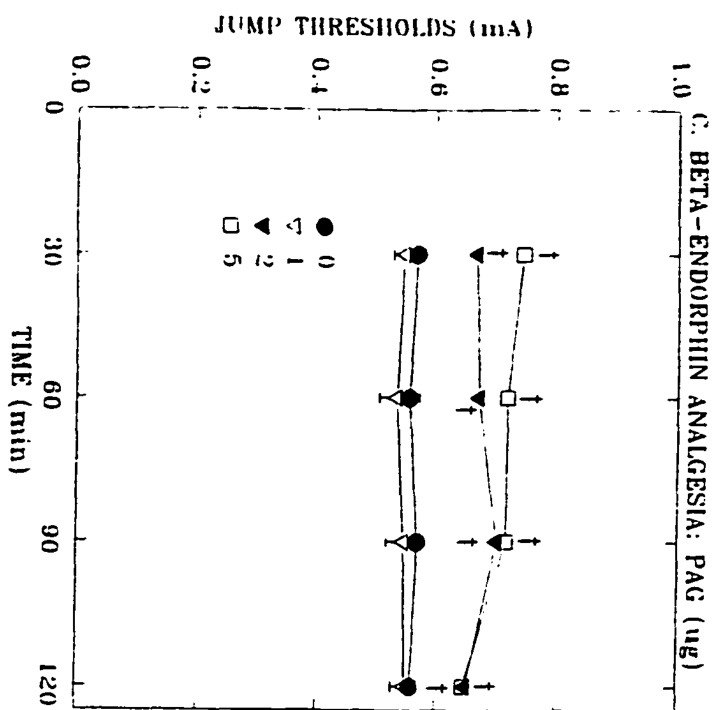

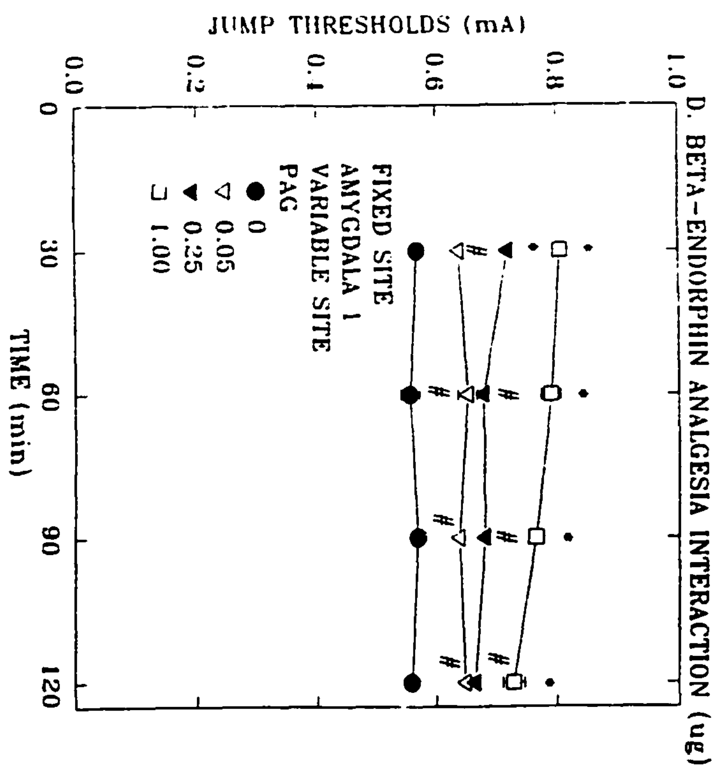

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endorphin doses in the PAG alone (Figure 12D). In contrast, beta-endorphin interactions between the amygdala and PAG failed to occur on the tail-flick test (Table IV).

Regional Interactions between Morphine and Beta-Endorphin Analgesia in the Amygdala and PAG: Significant differences were observed among experimental conditions (tail-flick: $F(6,231)= 31.20$, $p<.0001$; jump: $F= 121.24$, $p<.0001$), across test times (tail-flick: $F(3,693)= 79.23$, $p<.0001$; jump: $F= 85.97$, $p<.0001$) and for the interaction between conditions and times ($F(18,693)= 34.07$, $p<.0001$; jump: $F= 26.70$, $p<.0001$). As indicated previously, pairing morphine microinjections of 1 ug into the amygdala and 1 ug into the PAG produced significant increases in jump thresholds, indicating regional interactions between these sites in this condition. Further, as indicated previously, pairing beta-endorphin microinjections of 1 ug into the amygdala and 1 ug into the PAG also produced significant increases in jump thresholds, indicating regional interactions between the two sites in this condition. However, pairing microinjections of morphine (1 ug) in the amygdala with beta-endorphin (1 ug) in the PAG produced transient increases in jump thresholds that were significantly lower than either when morphine (1 ug) was administered simultaneously to the amygdala and PAG or when beta-endorphin (1 ug) was administered simultaneously to the amygdala and PAG (Figure 13A). These data indicate a failure to observe regional interactions for this condition. Likewise, pairing microinjections of beta-endorphin (1 ug) in the amygdala with morphine (1 ug) in the PAG significantly increased jump thresholds that were significantly lower than either when morphine (1ug) was adninistered simultaneously to the amygdala and PAG or when beta-endorphin (1ug) was administered simultaneously to the amygdala and

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FIGURE 13. Alterations (Mean, ±SEM) in jump thresholds in rats receiving morphine (M: 1 ug) into either the amygdala (A) or PAG (P) and beta-endorphin (B) into the other site as compared to either vehicle treatment, morphine delivered to the amygdala and PAG, or beta-endorphin delivered to the amygdala and PAG (Panel A). Alterations (Mean, ±SEM) in jump thresholds in rats receiving morphine (1 ug) and beta-endorphin (1 ug) into the amygdala or morphine and beta-endorphin into the PAG as compared to either vehicle treatment, morphine delivered to the amygdala and PAG, or beta-endorphin delivered to the amygdala and PAG (Panel B). Tukey comparisons ($p < .05$) revealed significant differences in jump thresholds for the interactive effects relative to vehicle treatment (crosses) that were significantly lower (*) than either morphine delivered to the two sites or beta-endorphin delivered to the two sites.

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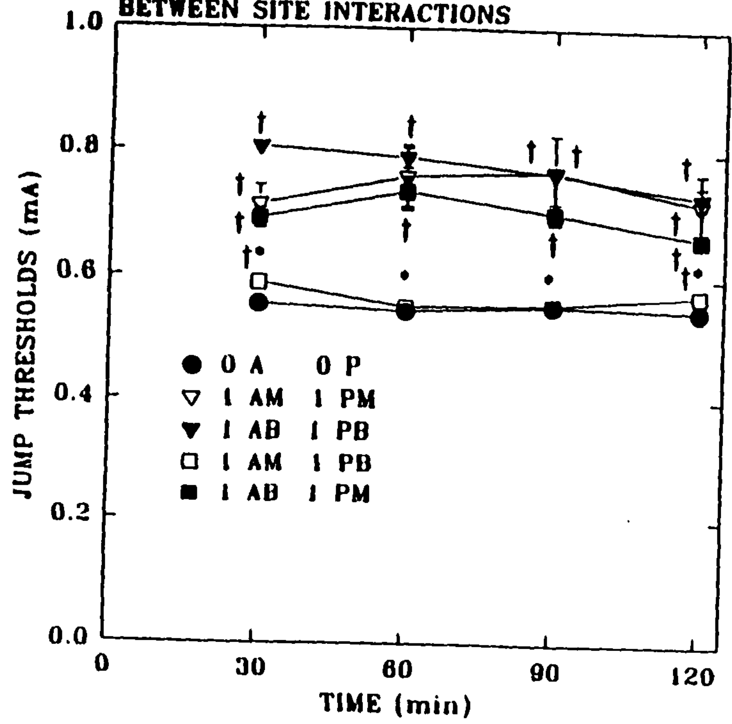

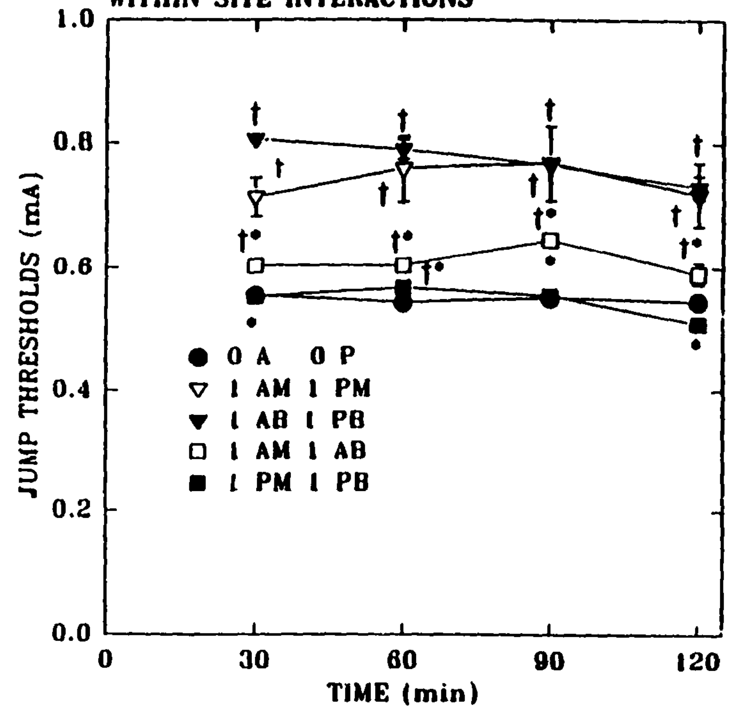

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PAG (Figure 13A). These data indicate smaller regional interactions for this condition. These experimental conditions were largely ineffective when the tail-flick test was employed, except for a small, but significant increase when beta-endorphin (1 ug) was administered into the amygdala and morphine (1 ug) was administered into the PAG (Table IV).

To examine whether the interactive effects of morphine (1 ug) and beta-endorphin (1 ug) into each of two sites were due to regional interactions, or due to simultaneous agonist administration, the two agonists were applied to a single site. Simultaneous administration of morphine (1 ug) and beta-endorphin (1 ug) into the amygdala significantly increased jump thresholds that were significantly lower than either when morphine (1 ug) was administered simultaneously to the amygdala and PAG or when beta-endorphin (1 ug) was administered simultaneously to the amygdala and PAG (Figure 13B). Similarly, simultaneous administration of morphine (1 ug) and beta-endorphin (1 ug) into the PAG significantly increased jump thresholds that were significantly lower than either when morphine (1 ug) was administered simultaneously to the amygdala and PAG or when beta-endorphin (1 ug) was administered simultaneously to the amygdala and PAG (Figure 13B). Again, these latter experimental conditions were largely ineffective when the tail-flick test was employed, except for a significant increase when morphine (1 ug) and beta-endorphin (1 ug) were administered simultaneously into the PAG (Table IV).

DISCUSSION

Morphine and Beta-Endorphin Analgesia: Test-Specific Effects: As expected, morphine and beta-endorphin produced potent dose-dependent analgesic responses in the

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PAG on the tail-flick and jump tests. In contrast, whereas microinjections of morphine and beta-endorphin into the amygdala produced orderly dose-dependent increases in jump thresholds, both agonists produced small effects on the tail-flick test. The increases in jump thresholds following opiate administration into the amygdala confirm previous results (Pavlovic et al., 1996; Rodgers, 1977, 1978). The small, inconsistent increases in tail-flick latencies following opiate agonists into the amygdala are less than those observed previously in awake (Pavlovic et al., 1996; Yaksh et al., 1976) and anesthesized (Helmstetter et al., 1993,1995; Tershner and Helmstetter, 1995) rats. These test-specific effects were also observed for regional interactions for morphine applied simultaneously to the amygdala and PAG, for beta-endorphin applied simultaneously to the amygdala and PAG, and for one agonist applied to one site and the other agonist applied to the second site in that increases were consistently noted on the jump test, but inconsistently on the tail-flick test. Such data underscore the importance of assessing analgesic measures with mulitple nociceptive tests. One compelling reason for failing to observe opioid analgesic effects in the amygdala on the tail-flick test may be the radiant heat level employed which elicited very short (< 3 sec) baseline latencies. Other studies observing opioid analgesia on the tail-flick test employed less intense heat levels which elicited longer (6 sec) baseline latencies (Helmstetter et al., 1993, 1995; Tershner and Helmstetter, 1995). This and other parametric variables (such as inter-test intervals) may have made the nociceptive stimulus more intense and thus more impervious to amygdala opioid microinjections. One previously-discussed (Pavlovic et al., 1996) possibility for such test-specific differences in the potencies of morphine and beta-endorphin analgesia elicited from the amygdala is the

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relative participation of the amygdala in differentially mediating each pain test at different levels of the neuraxis. Whereas the tail-flick test (D'Amour and Smith, 1941) is mediated at the level of the spinal cord based upon anatomical (Grossman, Basbaum and Fields, 1982) and behavioral (see reviews: Bodnar, 1986; Mayer and Manning, 1995; Terman et al., 1984) evidence, the jump test (Evans, 1961) is mediated by supraspinal mechanisms. One can observe the same pattern of differential and selective test-specific effects in other paradigms. For example, lateral hypothalamic electrical stimulation potently suppresses complex (e.g., escape, vocalizations) forms of nociception, but is either less effective or ineffective upon simpler (e.g., tail-flick, hot-plate) forms of nociception (Carr et al., 1982, 1985; Stellar et al., 1979; Yunger and Lorens, 1973). Thus, electrical stimulation of the lateral hypothalamus at non-reinforcing intensities will reduce escape behavior elicited by electrical stimulation of the nucleus reticularis gigantocellularis (Carr et al., 1982) in the same manner that systemic morphine reduces these escape responses (Carr et al.,1982). Lateral hypothalamic electrical stimulation also elevates the threshold for post-stimulus vocalization responses (Carr and Uysal, 1985), but not for thermal stimuli (Carr et al., 1982, 1985), suggesting that the lateral hypothalamus may operate as a gate for aversion information that has already been received by the central nervous system.

A second example is pregnancy-induced analgesia which is observed consistently and strongly when either the jump test (Baron and Gintzler, 1984, 1987; Gintzler, 1980; Gintzler et al., 1983; Sanders et al.,1988) or colorectal distension (Iwasaki et al., 1991) is used. In contrast, thermal stimuli produce either modest effects in indicating the presence of pregnancy-induced analgesia (Kristal et al., 1990). A second possibility is that the jump

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test may be measuring other factors in addition to pain sensitivity such as aversion. Since opioids can affect aversive responses (Bolles and Fanselow, 1980; Carr et al.,1982; Fanselow, 1984; Helmstetter and Fanselow, 1987; Lichtman and Fanselow, 1991) as well as pure nociceptive responses, it is possible that the increased jump thresholds observed by either opioid microinjections into the amygdala alone or opioid interactions between the amygdala and PAG may represent changes in aversive responsiveness as compared to nociceptive responsiveness. Such data indicating greater sensitivity to shock-reactive (or aversive) stimuli as compared to reflexive thermal stimuli to analgesic processes in the amygdala (or its interaction with the PAG) is also highly consistent with the amygdala's role in such mechanisms as fear, anxiety and conditioned emotional responses (see reviews: Davis, 1992; LeDoux, 1992).

Amygdala-PAG Interactions and Morphine Analgesia: The present study demonstrated that simultaneous administration of subthreshold doses of morphine into the amygdala and PAG significantly increased jump thresholds relative to administration of vehicle or low (1-2 ug) doses of morphine into either site alone. When a fixed subthreshold dose (1 ug) was applied to one site, morphine analgesia was observed at lower (0.05-0.25 ug) doses applied to the second site. Similar enhancements of morphine analgesia were observed regardless of which site received the fixed dose and which site received the variable dose. The observations of interactive effects on the jump, but not tail-flick tests was probably due to the failure of morphine to elicit consistent analgesia on the latter measure from the amygdala. It does not appear that these effects could be attributed to the diffusion of morphine between regions since analgesia elicited by paired

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(1 ug each) morphine treatment in the amygdala and PAG was significantly greater than analgesia elicited by morphine (2 ug) into either region alone.

Therefore, the multiplicative analgesic interactions between the amygdala and PAG for morphine observed in the present study are similar to those observed for spinal/supraspinal morphine synergy (Miyamoto et al., 1991; Pick and Pasternak, 1992, 1993; Roerig et al.,1984; Siuciak and Advokat, 1989; Yeung and Rudy, 1980), and to those observed for morphine synergy between the PAG and RVM, the RVM and LC, and the LC and PAG (Rossi et al.,1993). There are two possibilities for the site(s) of such interactions: a) through the anatomical circuitry between the amygdala and PAG (Basbaum and Fields, 1979; Da Costa Gomez et al.,1995; Helmstetter et al.,1993; Manning and Mayer, 1995), and/or b) through mechanisms initiated from these two sites converging at yet another site or sites. Support for the first possibility is derived from both pharmacological and neurophysiological data. General (naltrexone), $delta_2$ (NTII) and mu (BFNA, CTOP) opioid antagonists microinjected into the PAG reduce analgesia induced by either morphine, beta-endorphin or DAMGO microinjected into the amygdala (Pavlovic et al.,1996; Tershner and Helmstetter, 1995). The opioid modulation by the PAG of opioid analgesia in the amygdala is site-specific since opioid antagonists administered into the lateral mesencephalon fail to exert effects (Pavlovic et al.,1996). Neurophysiological evidence (Da Costa Gomez et al.,1995) indicates that electrical or chemical stimulation of the central nucleus of the amygdala produced excitatory or inhibitory effects in approximately 50% of identified PAG cells. A potential site of action for the second possibility of convergence is the RVM since: a. it receives anatomical connections from

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both the amygdala and PAG (Basbaum and Fields, 1979; Price and Amaral, 1981), b) it displays analgesic synergy with the PAG and LC (Rossi et al.,1993, 1994), and c) it mediates opioid analgesic effects elicited by the amygdala and PAG in lesion or localized antagonist studies (Helmstetter et al., 1997; Kiefel et al.,1993; Roychowdhury and Fields, 1996; Young and Mayer, 1984). At this point, it is not possible to state with certainty whether the interaction resides in intrinsic connections between the amygdala and PAG, or converges upon a third site like the RVM.

Amygdala-PAG Interactions and Beta-Endorphin Analgesia: The present study also demonstrated that simultaneous administration of subthreshold doses of beta-endorphin into the amygdala and PAG significantly increased jump thresholds relative to administration of vehicle or low (1-2 ug) doses of beta-endorphin into either site alone. The potency of beta-endorphin interactions was observed at very low (0.05 ug) doses applied to the variable site, and occurred regardless of which received the fixed dose and which site received the variable dose. Again, the observation of interactive effects for beta-endorphin on the jump, but not the tail-flick test was probably due to the minimal analgesia elicited by beta-endorphin from the amygdala. The present demonstration of the ability of beta-endorphin to produce multiplicative analgesic interactions between the amygdala and PAG parallels the abilities of other opioid agonists to produce synergistic effects as observed for beta-endorphin and DAMGO in spinal/supraspinal interactions (Roerig and Fujimoto, 1989; Roerig and Tseng, 1988), for ethylketocyclazocine in PAG/LC interactions (Bodnar et al.,1991), and for DAMGO and deltorphin II for PAG/RVM interactions (Rossi et al.,1994). Again, it is not possible to state with certainty

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whether the interaction resides in intrinsic connections between the amygdala and PAG, or convergence upon a third site.

Regional Interactions for Morphine and Beta-Endorphin Analgesia:

The present study found that multiplicative analgesic interactions occurred when either morphine was simultaneously applied to the amygdala and PAG, or when beta-endorphin was simultaneously applied to the amygdala and PAG irrespective of the site receiving the fixed dose and the site receiving the variable dose. This variable became important when morphine was applied to one site and beta-endorphin was applied to the other site simultaneously. If a subthreshold 1 ug dose of morphine was administered in the PAG simultaneously with a subthreshold 1 ug dose of beta-endorphin in the amygdala, a multiplicative analgesic interaction occurred that was comparable to applying either morphine to both sites or beta-endorphin to both sites. In contrast, if a subthreshold 1 ug dose of beta-endorphin was administered in the PAG simultaneously with a subthreshold 1 ug dose of morphine in the amygdala, jump thresholds failed to differ from vehicle treatment, and no multiplicative interaction was observed. The presence of a multiplicative interaction for the beta-endorphin: amygdala/ morphine: PAG combination is not surprising since it is consistent with a common mechanism of action for morphine and beta-endorphin in the amygdala. Previous studies have shown that analgesia elicited by morphine, mu receptor agonists and beta-endorphin in the amygdala can be blocked by administration of general, mu and $delta_2$ antagonists in the PAG (Pavlovic et al.,1996; Tershner and Helmstetter, 1995). Therefore, in this model, morphine or beta-endorphin applied to the amygdala activates a descending circuit to the PAG that activates an opioid

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(mu or $delta_2$) receptor. The multiplicative interaction paradigm would have subthreshold doses of morphine or beta-endorphin in the amygdala partially activating the descending circuit to the PAG that is activated further by the subthreshold dose of morphine applied locally in the PAG. Alternatively, the possibility exists that this interaction is dependent upon convergent actions upon a third site.

Why does the application of beta-endorphin in the PAG fail to activate a multiplicative interaction with morphine applied to the amygdala? First, this was somewhat surprising because of the suggestive presence of synergistic interactions between morphine and beta-endorphin in the PAG at higher doses (Smith et al., 1992). A compelling reason is that beta-endorphin in the PAG is activating a different neurochemical circuit than morphine. This possibility is supported by several lines of evidence. First, there are clear dissociations between analgesia elicited by ventricular morphine and beta-endorphin. Intrathecal naloxone blocked beta-endorphin, but not morphine analgesia (Tseng et al.,1987). Immunoreactive spinal met-enkephalin is released when beta-endorphin, but not morphine is administered ventricularly and hypothalamically (Tseng et al., 1985; Tseng and Wang, 1992). Antibodies to met-enkephalin and spinal delta antagonists block beta-endorphin, but not morphine analgesia (Suh and Tseng, 1990; Tseng and Suh, 1989). Ventricular morphine and beta-endorphin fail to develop analgesic cross-tolerance (Suh and Tseng, 1990), and are differentially altered by pentobarbital anesthesia (Tseng and Tang, 1992). Morphine and beta-endorphin only display additive analgesic effects following ventricular and intrathecal administration (Roerig and Tseng, 1988).

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Second, there are also differences between analgesic responses following morphine and beta-endorphin within the PAG. Barbiturate anesthesia reduces morphine analgesia, and enhances beta-endorphin analgesia in the PAG (Smith et al., 1992). Whereas beta-endorphin analgesia in the PAG is dependent upon a spinal opioid component, morphine analgesia in the PAG is dependent upon spinal noradrenergic and serotonergic receptors (Suh and Tseng, 1989; Suh et al., 1988; Tseng and Collins, 1991; Tseng and Tang, 1990). Although analgesia induced by either morphine or beta-endorphin in the PAG was blocked by either general (naltrexone) or mu-selective (CTOP) antagonists, the slopes of the dose-inhibition curves were not parallel (Monroe et al., 1996; Smith et al., 1992), suggesting the involvement of separate receptor subpopulations. In contrast, the opioid receptor antagonist, beta-endorphin$_{1-27}$, blocked both morphine and beta-endorphin analgesia from the PAG in similar fashion (Smith et al., 1992). Three distinct pharmacological mechanisms have been described to mediate beta-endorphin analgesia elicited from the PAG. Naltrexone and CTOP blocked analgesia induced by PAG beta-endorphin over a dose range which also inhibited analgesia to an equieffective analgesic dose of PAG morphine. However, one component of PAG beta-endorphin analgesia is characterized by an affinity for CTOP which is higher than that affected by morphine, and thus, CTOP blocked a major portion of PAG beta-endorphin analgesia over a dose range that did not affect the effect of an equianalgesic dose of morphine (Monroe and Smith, 1996). Thus, the mechanisms activated by the employed doses of beta-endorphin in the PAG do not appear to be convergent with the mechanisms activated by morphine in the amygdala, and this lack of convergence might fail to occur in the PAG or at a third site.

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V. EXPERIMENT III

INTRODUCTION:

The amygdala has been implicated in mu-receptor mediated analgesic responses. While microinjections of either mu opioid agonists (e.g., morphine, [D-Ala^2, N-Me Phe^4, Gly-ol^5]-enkephalin (DAMGO), beta-endorphin, an enkephalinase inhibitor, neurotensin or carbachol into the amygdala each elicit analgesia on the tail-flick test (Al-Rodhan et al., 1990; Helmstetter et al., 1993, 1995; Kalivas et al., 1982; Klamt and Prado, 1991; Pavlovic et al., 1996; Rodgers, 1978), morphine and beta-endorphin each produce analgesia on the jump test following amygdala microinjections (Pavlovic and Bodnar, 1997; Pavlovic et al., 1996; Rodgers, 1977, 1978). This latter nociceptive measure which assesses reactivity to shock (Evans, 1961) appears to be more sensitive to opioid agonist effects in the amygdala than the tail-flick test, which measures reactivity to heat (D'Amour, 1941). These test-specific differences may be due to the relative participation of the amygdala in differentially mediating the tail-flick and jump tests at different levels of the neuraxis (e.g., Bodnar, 1986; Grossman et al., 1982; Mayer and Manning, 1995; Terman et al., 1984). Alternatively, the jump test may be measuring other factors in addition to pain sensitivity such as aversion. Since opioids can affect aversive responses (Bolles and Fanselow, 1980; Carr et al., 1982; Fanselow, 1984; Helmstetter and Fanselow, 1987; Lichtman and Fanselow, 1991) as well as pure nociceptive responses, it is possible that the increased jump thresholds observed following morphine or beta-endorphin microinjected into the amygdala may represent changes in aversive responsiveness. Such data are highly consistent with the amygdala's role in such mechanisms as fear, anxiety and conditioned emotional responses (see reviews: Davis, 1992; LeDoux, 1992).

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While mu opioid agonists elicit analgesia from the amygdala, neither kappa (U50488H) nor delta ([D-Pen^2, D-Pen^5]-enkephalin (DPDPE)) agonists are capable of eliciting analgesia from the amygdala on the tail-flick test in barbiturate-anesthetized rats (Helmstetter et al., 1995). U50488H is a selective kappa$_1$ opioid agonist (Clark and Pasternak, 1988; VanVoigtlander et al., 1983; Zukin et al., 1988) capable of eliciting analgesia on the tail-flick (Gistrak et al., 1989; Millan, 1989; Millan et al., 1989; VanVoigtlander et al., 1983) and other nociceptive (Czlonkowski et al., 1987; Schmauss and Yaksh, 1984) tests following ventricular administration, but fails to produce analgesia on the tail-flick test following microinjection into such supraspinal sites as the PAG, RVM and theLC (Bodnar et al., 1991; Rossi et al., 1994). Since kappa opioid receptors have been implicated in the mediation of aversively-motivated behavior (DeRossett and Holtzman, 1985; Fanselow et al., 1989a, 1989b; Millan and Coelpaert, 1991), the first goal of the present study examined whether microinjections of the kappa$_1$ agonist, U50488H into the amygdala would elicit test-specific analgesia on the jump test relative to the tail-flick test. To assess the receptor specificity of this effect, the second goal of the study examined whether U50488H-induced analgesia on the jump test elicited from the amygdala was blocked by pretreatment with the kappa$_1$ antagonist, Nor-BNI (Portoghese et al., 1987) administered into the same site. An opioid synapse in the PAG is integral for the full expression of morphine, DAMGO and beta-endorphin analgesia elicited from the amygdala given the effectiveness of general (naltrexone), mu (BFNA), and delta$_2$ (NTII) opioid antagonists as well as lidocaine administered into the PAG to reduce opioid analgesia elicited from the amygdala (Helmstetter et al., 1997; Pavlovic et al., 1996;

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Tershner and Helmstetter, 1995). Thus, the third goal of the study was to determine whether U50488H-induced analgesia on the jump test elicited from the amygdala was affected by PAG pretreatment with either naltrexone, BFNA or NTII.

This experiment has been accepted for publication in the journal, Analgesia.

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PROCEDURES

Figures 14 and 15 summarize the number and sample sizes of the different experimental groups In all conditions, tail-flick latencies and jump thresholds were determined 30, 60, 90 and 120 min following the amygdala microinjection. Animals receiving particular antagonist doses were matched on the basis of their basal nociceptive latencies and thresholds as well as the magnitude of U50488H-induced analgesia on the jump test at a 5 ug dose in the amygdala. There was a one-week interval between particular treatment conditions; this interval minimized potential agonist-induced tolerance effects and long-term antagonist effects as described in previous studies (Bodnar et al., 1991; Pavlovic et al., 1996; Rossi et al., 1994).

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RESULTS

Histological Verification: Histological verification revealed cannula placements in the amygdala which were located in the medial (n=9), basolateral (n=7), basomedial (n=6), central (n=8) and posterior basolateral (n=5) nuclei. Rats in both antagonist protocols had rostral cannula placements in the above nuclei of the amygdala, and evaluation of the overall magnitude of U50488H-induced analgesia on the jump test revealed no significant differences between these nuclei. Therefore, data for each agonist treatment for the two amygdala placements were pooled for antagonist analysis. PAG cannulae were located in the lateral (n=6) and ventral (n=5) PAG at the level of the III cranial nerve, the dorsal raphe nucleus (n=6), and the lateral (n=8) and ventrolateral (n=10) PAG at the level of the dorsal raphe nucleus. PAG placements were spread across antagonist treatments which did not differ as a function of such placements.

U50488H in the Amygdala: Tail-Flick Test: Significant differences in tail-flick latencies were observed among experimental conditions ($F(3,216)= 7.29$, $p<.0003$), across test times ($F(3,204)= 7.24$, $p<.0001$) and for the interaction between conditions and times ($F(9,204)= 4.51$, $p<.0001$). U50488H produced transitory but significant increases in tail-flick latencies following the 2 (90 min) and 5 (120 min) ug doses in the amygdala (Figure 14A). Each of these effects were small in magnitude, increasing latencies by less than 0.5 sec.

U50488H in the Amygdala: Jump Test: Significant differences in jump thresholds were observed among experimental conditions ($F(3,68)= 54.76$, $p<.0001$),

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FIGURE 14. Alterations (Mean, ±SEM) in tail-flick latencies (Panel A) and jump thresholds (Panel B) following microinjections of the $kappa_1$ opioid agonist, U50488H into the amygdala at doses of 0 (n=26), 1 (n=7), 2 (n=9) and 5 (n=9) μg. Panel C indicates alterations in U50488H-induced (5μg : n=9) analgesia on the jump test elicited from the amygdala relative to either vehicle treatment (n=18), or following preatretment with the $kappa_1$ opioid antagonist, nor-binaltorphamine (Nor-BNI: 0.1μg, n=6; 1μg, n=6; 4 μg, n=6) administered into the amygdala. The daggers in this and subsequent figures indicate significant (Tukey comparisons, $p<.05$) differences in jump thresholds relative to vehicle treatment. The stars in this and subsequent figures indicate significant ($p<.05$) differences in jump thresholds following antagonist preatretment relative to vehicle/U50488H treatment.

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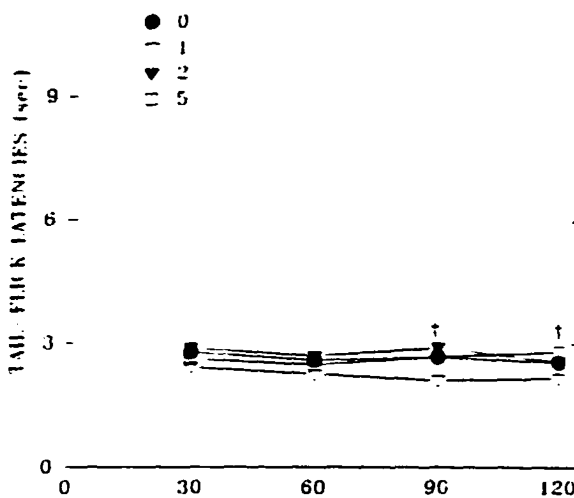

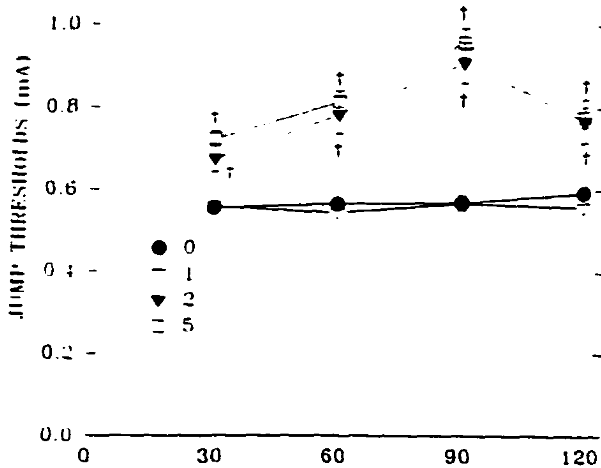

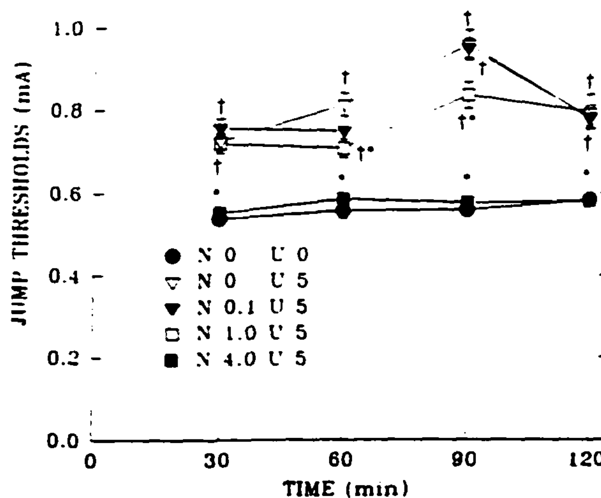

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across test times ($F(3,204) = 70.33$, $p < .0001$) and for the interaction between conditions and times ($F(9,204) = 21.20$, $p < .0001$). U50488H produced significant increases in jump thresholds across the entire 120 min time course following the 2 and 5, but not the 1 ug doses in the amygdala (Figure 14B). In contrast to the concomitant changes in tail-flick latencies, the changes in jump thresholds were dose-dependent and robust, equivilent in potency to that previously observed for morphine and beta-endorphin (Pavlovic and Bodnar, 1997; Pavlovic et al., 1996; Rodgers, 1977, 1978).

Nor-BNI and U50488H Analgesia in the Amygdala: Significant differences in jump thresholds were observed among experimental conditions ($F(4,80) = 101.46$, $p < .0001$), across test times ($F(3,240) = 170.75$, $p < .0001$) and for the interaction between conditions and times ($F(12,240) = 34.44$, $p < .0001$). The significant increases in jump thresholds across the time course following amygdala microinjections of U50488H were significantly and dose-dependently reduced by pretreatment with Nor-BNI in the amygdala (Figure 14C). Both the low (0.1 ug, 60 min) and middle (1 ug, 60-90 min) doses of Nor-BNI produced transitory, but significant reductions in U50488H-induced analgesia in the amygdala. The high (4 ug) dose of Nor-BNI completely eliminated U50488H-induced analgesia across the time course.

Opioid Antagonists in the PAG and U50488H Analgesia in the Amygdala: Significant differences in jump thresholds were observed among experimental conditions ($F(7,152) = 90.83$, $p < .0001$), across test times ($F(3,456) = 329.78$, $p < .0001$) and for the interaction between conditions and times ($F(21,456) = 95.57$, $p < .0001$). The significant increases in jump thresholds across the time course following amygdala microinjections of

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U50488H were significantly and dose-dependently reduced by pretreatment with naltrexone in the PAG across the time course with the 1 and 5 ug doses producing overall respective reductions of 38% and 87% (Figure 15A). U50488H-induced analgesia in the amygdala was also significantly and dose-dependently reduced by pretreatment with BFNA in the PAG with the 1 and 4 ug doses producing overall respective reductions of 42% and 74% (Figure 15B). In contrast, U50488H-induced analgesia in the amygdala was transiently though significantly reduced by pretreatment with NTII in the PAG following the 1 (90-120 min) and 4 (60-90 min) ug doses (Figure 15C).

DISCUSSION

U50488H-Induced Analgesia in the Amygdala: The $kappa_1$ opioid agonist, U50488H produced a test-specific and dose-dependent analgesic effect on the jump test, but not the tail-flick test following microinjection into the amygdala. While amygdala microinjections of U50488H produced transient (<0.5 sec) increases in tail-flick latencies, the same dose range (1-5 ug) significantly increased jump thresholds during the same test period and over the entire time course. These increases in jump thresholds following amygdala microinjections of U50488H were similar in potency (1-5 ug), magnitude (approximately 50-70% increases in thresholds), duration (120 min) and peak effect (60-90 min) as was observed for morphine and beta-endorphin (Pavlovic and Bodnar, 1997; Pavlovic et al., 1996). Interestingly, ventricular administration of kappa agonists failed to increase the threshold to elicit vocalizations to high, supra-threshold levels of electric tail shock (Millan, 1989, 1990), suggesting either that the agonist must be placed directly into the amygdala to elicit shock reactivity changes, or that kappa agonists alter reactivity to

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FIGURE 15. Alterations (Mean, ±SEM) in in jump thresholds following microinjections of either vehicle (n=21) or the kappa$_1$ opioid agonist, U50488-H into the amygdala (n=9) in rats pretreated with either the general opioid antagonist, naltrexone(Panel A: Ntx; 1μg , n=6; 5μg, n=6), the mu opioid antagonist, BFNA (Panel B: BFNA; 1μg, n=6; 4μg, n=6), or the delta2 opioid antagonist, NTII (Panel C: NTII; 1μg, n=6; 4μg, n=6), administered into the PAG.

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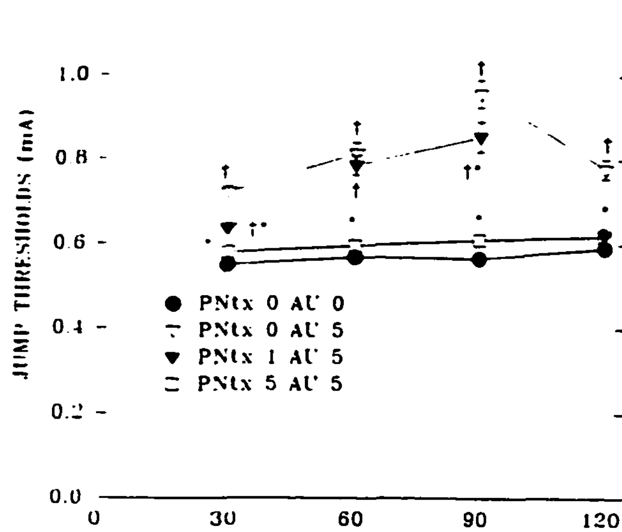

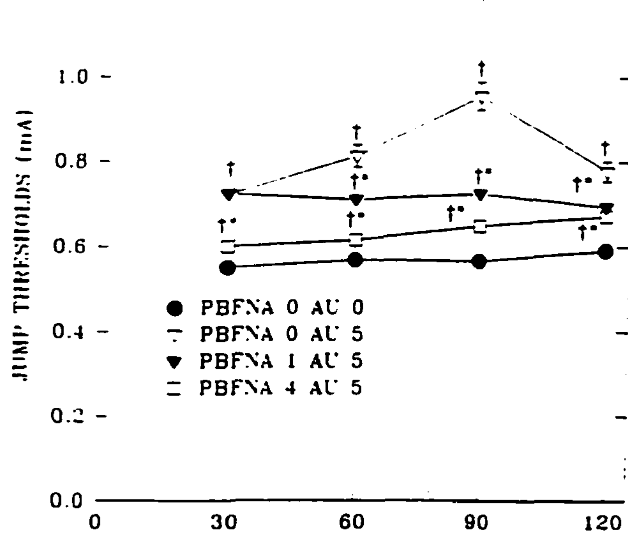

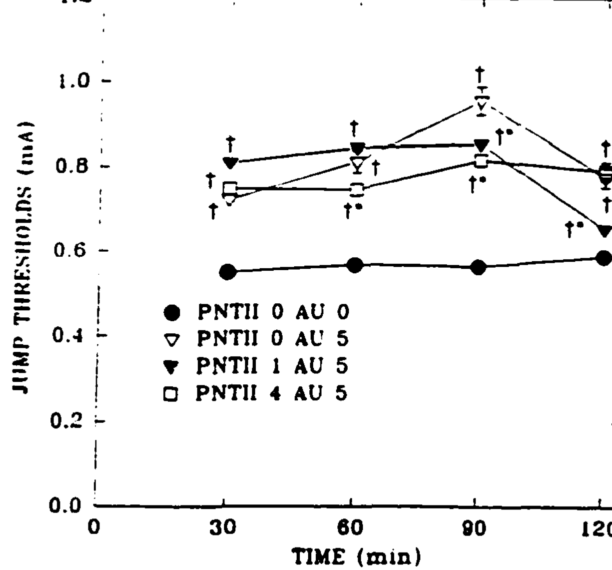

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shock at threshold, but not suprathreshold levels. The failure of U50488H to increase tail-flick latencies following amygdala microinjections is in agreement with the failure to observe effects in barbiturate-anesthetized rats tested at longer (6 sec) baseline latencies (Helmstetter et al., 1995), and the failure of kappa agonists to produce analgesia following ventricular administration at low latencies induced by high-intensity radiant heat (Millan, 1989, 1990). As introduced in the rationale for the present study, these test-specific effects could be attributed to either the relative participation of the amygdala in differentially mediating the tail-flick and jump tests at different levels of the neuraxis, or alternatively, the ability of the jump test to measure other factors in addition to pain sensitivity such as aversion. Such data are highly consistent with the amygdala's role in such mechanisms as fear, anxiety and conditioned emotional responses (see reviews: Davis, 1992; LeDoux, 1992), as well as roles of the kappa opioid receptors in the mediation of aversively-motivated behavior (DeRossett and Holtzman, 1985; Fanselow et al., 1989a, 1989b; Millan and Coelpaert, 1991). Since the tail-flick test was the only nociceptive measure employed in evaluating the ability of $kappa_1$ receptor agonists to elicit analgesia from other supraspinal structures like the PAG, LC and RVM (Bodnar et al., 1991; Rossi et al., 1994), it will be important to re-evaluate whether U50488H is effective in producing analgesia from these sites using other nociceptive measures, including the jump test.

Nor-BNI and U50488H-Mediated Effects: The $kappa_1$ receptor specificity of U50488H-induced analgesia in the amygdala was confirmed by showing that this response was significantly and dose-dependently reduced by pretreatment with the $kappa_1$ opioid antagonist, Nor-BNI in the amygdala. However, Nor-BNI is a $kappa_1$ opioid antagonist

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(Portoghese et al., 1987) that has some long-acting (Horan et al., 1992; Jones and Holtzman, 1992; Paronis et al., 1993) and chronic (Spanagel et al., 1994) actions at mu opioid receptors. It should be noted that Nor-BNI was 6-fold more potent in blocking U50488H-induced analgesia than morphine-induced analgesia, while systemic naltrexone (0.2 mg/kg) reversed morphine-, but not U50488H-mediated analgesia (Millan et al., 1989). Given these provisos, the effectiveness of Nor-BNI in reducing U50488H-induced analgesia in the amygdala can certainly specify opioid involvement, but one must proceed with caution in specifically delineating the $kappa_1$ opioid receptor subtype. A further study may be able to specify opioid receptor subtype involvement in this response by using antisense oligodeoxynucleotides directed against the KOR-1 clone which specifically reduced kappa agonist-induced analgesia (Adams et al., 1994; Chien et al., 1994).

PAG Opioid Antagonists and U50488H-Induced Analgesia in the Amygdala: The present study indicated that an opioid synapse in the PAG was integral for the full expression of U50488H-induced analgesia elicited from the amygdala on the jump test. The general opioid antagonist, naltrexone in the PAG reduced U50488H-induced analgesia in the amygdala by 87%. These effects persisted in both lateral, ventrolateral and ventral PAG placements, and blocked analgesia elicited from central and baso-lateral amygdala nuclei. Therefore, PAG naltrexone's effect upon U50488H-induced analgesia in the amygdala parallelled its actions upon analgesia elicited by either DAMGO, morphine or beta-endorphin microinjected into the amygdala (Pavlovic et al., 1996; Tershner and Helmstetter, 1995). A previous (Pavlovic et al., 1996) study has indicated the specificity of this effect since naltrexone in misplaced control sites in the lateral mesencephalon was

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ineffective in reducing analgesia elicited from the amygdala, and since the small, sporadic hyperalgesia elicited by naltrexone in the PAG could not account for its antagonistic actions upon agonist-induced analgesia elicited from the amygdala.

There appears to be some subtle differences in the potential opioid receptor subtypes involved in the opioid synapse in the PAG that mediate opioid agonist effects in the amygdala. The mu antagonist, BFNA in the PAG produced small (11%) reductions in morphine analgesia on the jump test in the amygdala, and failed to alter beta-endorphin analgesia on the jump test in the amygdala (Pavlovic et al., 1996). In contrast, the mu antagonist, BFNA reduced U50488H-induced analgesia on the jump test in the amygdala by 74%. This differential effect occurred despite the fact that the three agonists produced approximately equi-analgesic effects in the amygdala. The effects with the $delta_2$ antagonist, NTII yielded a different pattern of effects. Whereas NTII in the PAG significantly reduced analgesia induced by amygdala microinjections of either morphine (60%) or beta-endorphin (79%) on the jump test, NTII in the PAG produced small (16%) reductions in U50488H-induced analgesia on the jump test in the amygdala. These data taken together suggest that the mu, but not the $delta_2$, receptor in the PAG is important in relaying this putative kappa-mediated response in the amygdala.

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VI. GENERAL DISCUSSION

A. Summary of Findings: The present dissertation examined analgesic potencies of different agonists following their microinjection into the amygdala, as well as the functional relationship between the amygdala and PAG in the processes of pain and analgesia. In order to examine the functional relationship between the amygdala and the PAG we utilized two approaches: agonist-antagonist and synergy paradigms. The following conclusions are summerized as follows:

1. Both morphine (2.5-5 ug) and beta-endorphin (2.5-5 ug) microinjected in either the baso-lateral or central nuclei of the amygdala significantly increased tail-flick latencies and jump thresholds in rats. The increases were far more pronounced on the jump test than on the tail-flick test. Placements dorsal and medial to the amygdala were ineffective.

2. Naltrexone (1-5 ug) in the PAG significantly reduced both morphine (tail-flick: 70-75%; jump: 60-81%) and beta-endorphin (tail-flick: 100%; jump: 93%) analgesia elicited from the amygdala, indicating that an opioid synapse in the PAG was integral for the full expression of analgesia elicited from the amygdala by both agonists.

3. Both BFNA (68%) and Ntii (100%) in the PAG significantly reduced morphine, but not beta-endorphin analgesia in the amygdala on the tail-flick test. Ntii in the PAG was more effective in reducing morphine (60%) and beta-endorphin (79%) analgesia in the amygdala on the jump test than BFNA (15-24%). These data indicate that PAG $delta_2$, and to a lesser degree mu opioid receptors are necessary for the full expression of morphine and beta-endorphin analgesia elicited from the amygdala. When opioid antagonists were microinjected in

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misplaced mesencephalic sites they did not block amygdaloid opioid analgesia, thus showing the specificity and importance of the PAG.

4. Co-administration of subthreshold doses of morphine (0.05-1ug) in both the amygdala and PAG results in a profound synergistic interaction on the jump test, but not the tail-flick test.

5. Co-administration of subthreshold doses of beta-endorphin (0.05-1ug) in both structures also results in a profound test-specific synergistic interaction. In both cases, the magnitude of the interaction was similar regardless of the site receiving the fixed dose of the opioid, and the site receiving the variable dose of the opioid.

6. Co-administration of beta-endorphin (1 ug) in the amygdala and morphine (1 ug) in the PAG produced a potent synergistic interaction.

7. Co-administration of morphine (1 ug) in the amygdala and beta-endorphin (1 ug) in the PAG failed to produce interactive effects. This results argues for the multiple modulatory mechanisms mediating beta-endorphin analgesia in the PAG, which is not the same as for the morphine.

8. Co-administration of morphine (1 ug) and beta-endorphin (1 ug) in either the amygdala alone or the PAG alone failed to produce an interaction, indicating the importance of regional opioid activation.

9. U50488H (kappa$_1$ agonist) microinjection in the amygdala dose-dependently (1-5ug) increased jump thresholds, but not tail-flick latencies. This analgesia was significantly and dose-dependently blocked by pretreatment with putative kappa$_1$ opioid antagonist Nor-BNI (0.1- 4ug) into the amygdala.

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10. PAG administration of either general (naltrexone: 1-5 ug) or mu (BFNA: 1-4ug) opioid antagonists significantly and dose -dependently blocked U50488H- induced analgesia on the jump test in the amygdala.

11. PAG administration of a $delta_2$ (NTII: 1-4ug) opioid antagonist was ineffective in reducing this response. Thus, U50488H produces a test-specific analgesic response following amygdala microinjections which is putatively mediated through a $kappa_1$ synapse in the same structure, and is dependent upon an opioid (potentially mu) synapse in the PAG for its full expression.

The fact that U50488H induced analgesia from the amygdala is mediated through PAG mu receptor, while morphine and B-endorphin are primarily using δ_2 opioid receptor in the PAG, is additional evidence for multiplicity of the endogenous nociceptive circuitries that also exist between the amygdala and the PAG.

B. Time course of Agonist Action: It was observed that agonist doses employed in our studies typically produced a peak effect in the amygdala after 60-90 min., while the same agonists produced peak effect sooner (15-30 min.) in the PAG, LC and RVM. There are several potential explanations for this effect:

1. The amygdaloid nuclei occupy much larger areas as compared to relevant brain stem structures as the PAG. In the amygdala, opioid receptors are dispersed throughout all nuclei with much higher densities in the centro-medial, as compared to the baso-lateral nuclei. The PAG nucleus is small, with high-density receptors tightly packed together. This pharmacokinetic difference alone could account for different timing of the peak effect.

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2. The lack of precision of microinjection of drug quantities into only one nucleus as opposed to others, due to the dispersion effect, could partially explain the delayed analgesic peak effect in the amygdala. The dispersion effect could also account for the fact that in our studies analgesic potencies of drugs did not differ as a function of place (BL versus CM amygdaloid nuclei).

3. Such pharmacokinetic and pharmacodynamic issues as absorption and sequestration of drug in tissue and opioid receptor occupancies,that could be specific to the amygdala and thereby different from those present in brainstem structures, might also explain the differential peak analgesic effects in the amygdala.

Another interesting observation is that the same (5 μg) dose of each agonist in the amygdala produced approximately an equipotent analgesic effect on the jump test. In the light of the fact that the amygdala is projecting to the RVM, either directly and/or through the PAG, it is probable that the behavioral effect is produced no matter what opioid agonist is microinjected into the amygdala, as long as they consistently activate "off-cells" in the RVM (Fields et al., 1991). It is known that a supression of "on-cell" firing in the RVM is not in itself sufficient to yield a behaviorally measureble antinociception (Heinricher and Fields, 1994). Only opioid microinjection that activate "off-cells" in the RVM highly correlates with the inhibition of nociception. Therefore, there is no high correlation between the type of the opioid agonist in the amygdala and the behavioral effect, but rather the activation of the same effector system (e.g. "off-cells" in the RVM) and the behavioral effect (e.g. analgesia) that are highly correlated with each other.

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Based on other studies and our previous experience, we made certain assumptions about drug-dose choises, their biochemical binding affinities, as well as about the time course of analgesic responses in the amygdala as well as in the PAG. We used the time course of 120 min. in the present studies to record changes in the baseline latencies as a measure of analgesic responses. The shape of our dose-response curve is such to indicate that after 120 min. nociceptive latencies are beginning to approach the baseline latencies. However, the small but still present analgesia after 120 min. does not allow us to make definitive statements about full area under the curve analyses.

C. Nociceptive Tests as Conditioned or Unconditioned Stimuli: The tail-flick and jump threshold tests that were employed to record analgesic responses employ unconditioned stimuli. In the early phases of determining the baseline latencies, tail-flick and jump threshold latencies were higher than subsequent tests, due to handling which can produce stress induced analgesia (SIA). However, with repeated handling adaptation occurred and, at the time of experimental testing, baseline latencies and thresholds were stable. It should be noted that amygdala lesions fail to change baseline tail-flick and jump threshold latencies (Pavlovic et al., 1996). In addition, exposure to the testing aparatus itself does not produce an analgesic response. Therefore, nociceptive stimuli produced unconditional analgesic responses following opiates microinjections and probably excluded any interaction of the drugs with conditioned analgesia.

D. Synergy Studies: The presence of synergy observed between the amygdala and the PAG indicates that a limbic lobe structure (e.g. amygdala) can interact with another

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brainstem structure (e.g. PAG) to produce an analgesic response. These studies have shown that not only site-site (the amygdala and the PAG) and drug-drug (e.g. morphine-morphine; B-endorphin-B-endorphin; B-endorphin-morphine) analgesic interactions were present, but also the drug/site-drug/site variable becomes integral in producing an analgesic response. Thus, when B-endorphin (1 μg) in the PAG was paired with morphine (1 μg) in the amygdala analgesic interactions did not occur. Combination of different variables (drug-drug and drug/site-drug/site) that become important in the synergistic relationship between the amygdala and the PAG can increase the variety of the behavioral responses in which pain is an important and constituent part, and, thereby increase the efficiencies of analgesic responses. Pain is an integral part of overall defensive reactions, as wel as in mechanisms of fear and anxiety in which the amygdala plays a pivotal and essential part. The synergistic relationship observed between the amygdala and the PAG for morphine and B-endorphin analgesia can serve the role of connecting and integrating the pain mechanism with all of these above-mentioned processes. It is not known whether cognitive and motivational components are conveyed to the PAG through the amygdala or through some other forebrain structure that projects directly to the PAG.

E. Future directions and implications: Most of the research on pain and analgesia delineates antinociceptive circuitry that is organized in rostro-caudal direction, starting with the PAG. Less research is directed towards forebrain structures, such as the amygdala, that could be either part of this antinociceptive circuitry and/or have an ability to modulate this circuitry.

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Morphine and beta-endorphin antinociceptive synergistic interaction between the amygdala and PAG parallels and complements the other synergistic interaction among supraspinal loci, such as PAG-RVM, PAG-LC and LC-RVM. The test specific results and dissociation of agonistic and synergistic potencies of morphine and beta-endorphin confirm Tseng's (Tseng et al., 1985, 1987, 1992), Smith's (Smith et al., 1992, 1996) and Fields' (Fields and Basbaum, 1992) work about multiplicity of endogenous antinociceptive circuitries. Not only are morphine and B-endorphin utilizing different pathways in mediating their analgesic responses, but their functional projections from the PAG to the spinal cord are multiple in nature with different underlying physiological mechanisms (Fields and Basbaum, 1992). The functional synergistic antinociceptive interaction between the amygdala and PAG further confirms utility of their mutual anatomical connections and is in line of evidence that chemical and physiological manipulations performed in one structure (e.g. the amygdala) can affect physiological processes in another structure (e.g. PAG). For example, in our synergy study, fixed subeffective doses of opioid agonists into either the amygdala or the PAG increases analgesic potency of subeffective doses of opioid agonists administered into another structure. The presence of the amygdala-PAG synergistic interaction may serve the purpose of speeding up the behavioral response, be it fight or flight. The enhancement of analgesic response by the amygdala's involvement can very well fit into whatever defensive mechanism is at hand, and therefore increase the probability for the animal's survival.

Both the amygdala and PAG are implicated in defensive behavior, and the amygdala has an undisputed role in the appraisal of danger, fear, anxiety, neuroendocrine responses to

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stress and emotional memory. Therefore, it could very well be that the amygdala is involved in analgesic processes in completely different ways and for different reasons than brainstem structures. It is possible that imminent danger which has obvious survival value activates a "quick-and-dirty" subcortical pathway in which the amygdala may have no big role (LeDoux, 1995). For example, body injury, at least in the initial stage, would not require the amygdala's involvement in the producing analgesic responses. The PAG alone or in the combination with other brainstem structure (the LC or the RVM) would produce an analgesic response. The exclusion of the amygdala would shorten the reaction time and therefore, increase the animal's chanse of the surviving. Although speculative, it is possible that in the course of time, the amygdala's modulation (e.g. enhancement through the synergy mechanism, inhibition or completly independent involvement) of the PAG's participation in the analgesic processes could be dependent on the situational context. Once cognitive factors become more important in establishing the context within which an emotional stimulus (such as danger) is to be evaluated, the amygdala's role become essential and gradation of the PAG's activation can determine the level of produced analgesia. This pattern of gradual co-activation,exclusion, synchrony or bigger role on one structure depending on the situational context that may operate between the amygdala and PAG in the processes of pain and analgesia, may very well be the mechanism involved in other defensive processes, in addition to pain.

A series of studies could be conducted to further delineate the amygdala's role in analgesic processes, as well as its nature of the relationship with other brainstem structures known to mediate analgesic processes. Future research can investigate whether a similar

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synergistic relationship exists between the amygdala and the RVM. It could be interesting to observe the nature of this possible functional analgesic relationship in the presence, versus absence of PAG involvement. Innactivation of the PAG could be achieved either through reversible or irreversible lesions, or application of opioid antagonists. Also, it would be useful to observe whether amygdala-LC synergy exists for morphine and B-endorphin, and whether the nature of this synergistic relationship would be the same as the one between the amygdala and the PAG, as well as the putative one between the amygdala and the RVM. The natural extension of these kind of studies would be to systematically try combination of other agonists between these pairs of structures, as well as to see whether subefective doses of opioid agonists microinjected into the amygdala can change the opioid synergy between the PAG and the RVM, the PAG and the LC, and the LC and the RVM. The ability of opioid agonists in the amygdala to change the nature of synergy in some of these brainstem structures would argue for a further modulatory role of the amygdala in analgesic processes.

In order for the amygdala to be activated, a certain level of arousal needs to occur. It is known that activation of the LC, the RVM and the VTA with subsequent and respective release of NE, 5HT and DA are essential parts of this arousal system. The aroused state, in turn, is a necessary condition for cognitive processing in which information about pain could be used to initiate an appropriate defensive reaction, or to activate the memory system in which remembering the particular stressful or painful event could be important in the future similar situations. If opioid agonists (in addition, or, in synchrony with GABA agonists) regulate the release of DA, NE and 5HT, it is, then, to be

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expected that subeffective opioid agonist doses given simultaneously in all of these three regions will produce different effects as compared to single-site injections. These effect will be best captured in some memory tasks in which the role of the amygdala could be determined according to its participation in active versus passive coping (e.g. avoidance tasks in which a painful stimuli are used as unconditional stimuli). An experiment could be disigned in which efficacies of opioid agonists administered into pairs of sites could be determined upon the amygdala's involvement in the memory tasks. It could be further hypothesized that efficacies of opioid agonists doses in memory tasks will be greater when they are microinjected into the amygdala, the LC or the RVM, or in the combination of these three, rather than into the amygdala and the PAG.

Sensitization involves strengthening of the connection between the amygdala and the brainstem structures, as well as better encoding and more efficient exchange of information between these structures. Therefore, opioids efficacy, measured by their negative impact on learning and memory, is expected to be better in retension phase, as opposed to acquisition phase of learning.

The above mentioned kind of studies could further delineate opioid agonists role in the amygdala's involvement in learning and memory processes.

If the importance of the amygdala as a structure in the mammals hierarchy was determined by the diversity and importance of the danger signals in the species environment, than we would expect that the amygdala's true role in mediating stress and analgesic responses will be discovered in the paradigms that are using dangerous stimuli present in the actual environment. While the amygdala's role in analgesic processes would

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be discovered when rats are exposed to agressive conspecifics or a natural predator (e.g. a cat), cold water swim paradigm that we used in one of our previous studies would not be appropriate because it is missing " a real life elements"(see Pavlovic and Bodnar, 1996). This factor should be also accounted for while designing experimental paradigms.

If conducted, the above mentioned suggestions and indications of areas of the research that can use and build up on our studies, can further enhance our knowledge about amygdala's role in mediation of pain and analgesia, fear and anxiety, as well as learning and memory processes.

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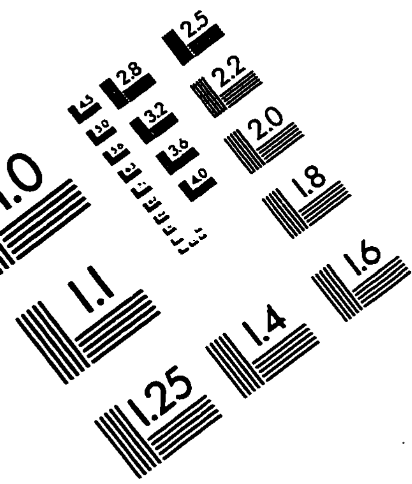
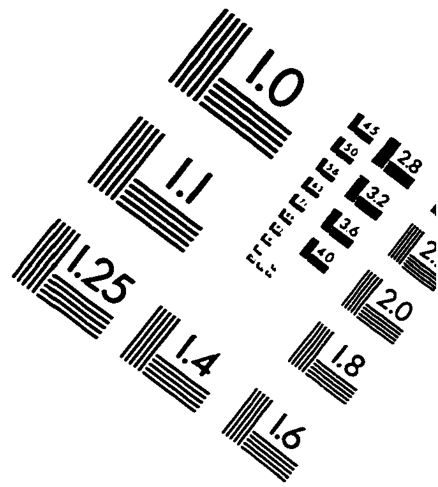
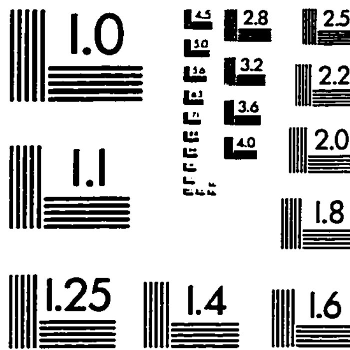
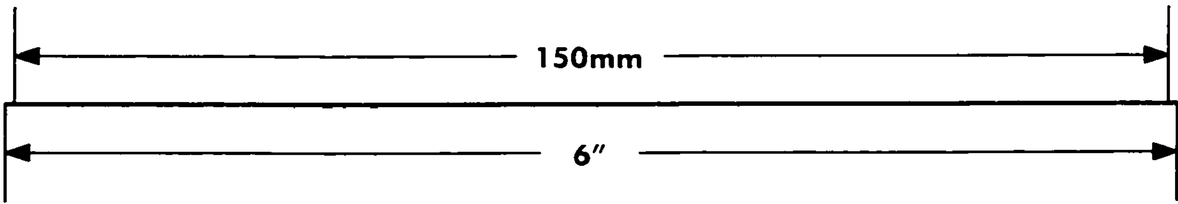
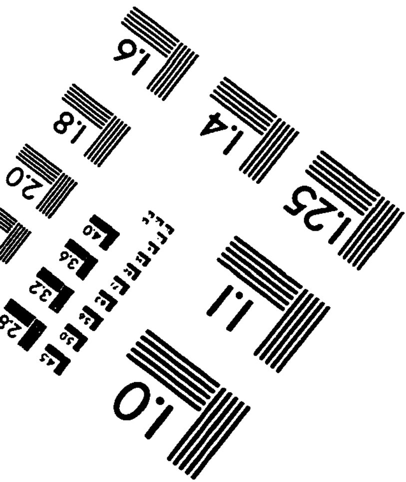
IMAGE EVALUATION TEST TARGET (QA-3)











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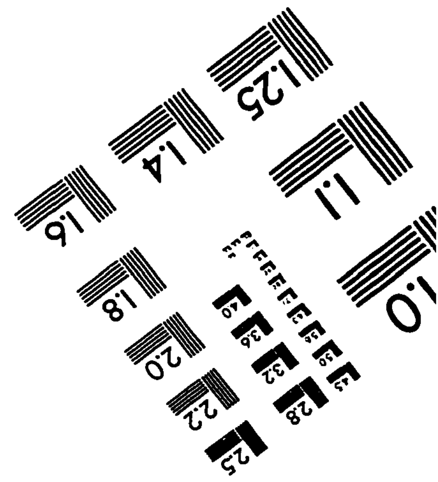

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