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# Supraspinal opiate antinociception: Synergy between mesencephalic, pontine and medullary sites in the rat

Rossi, Grace C., Ph.D.

City University of New York, 1993

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# SUPRASPINAL OPIATE ANTINOCICEPTION: SYNERGY BETWEEN MESENCEPHALIC, PONTINE AND MEDULLARY SITES IN THE RAT

by

GRACE C. ROSSI

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.

1993

c 1993

GRACE C. ROSSI

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## Abstract

Supraspinal Opiate Antinociception: Synergy between Mesencephalic, Pontine and Medullary Sites in the Rat

by

Grace C. Rossi

Mentor: Dr. Richard J. Bodnar

Morphine produces potent antinociception when injected into the periaqueductal gray (PAG), the rostral ventral medulla (RVM: nuclei raphe magnus and reticularis gigantocellularis), and the dorsolateral pons (DLP) which includes the locus coeruleus. Supraspinal opioid antinociception is mediated by intrinsic connections among these structures. Simultaneous administration of sub-antinociceptive doses of morphine into pairs of these three sites elicited dramatic antinociception, implying synergy. The most effective combination was the PAG and RVM; the PAG/DLP and RVM/DLP combinations were less efficacious. The marked synergy between the PAG and the RVM was sensitive to naloxonazine, implying a role for $mu_1$ receptors. Inclusion of a low morphine dose in one region produced significant leftward shifts in the other's antinociceptive dose response curve.

This dissertation then characterized the opioid receptor subtypes involved in the multiplicative

antinociceptive interaction between the PAG and RVM through the use of agonists of mu (D-Ala$^2$, Met-Phe$^4$, Gly(ol)$^5$-enkephalin: DAMGO), kappa (U50488H), delta$_1$ (D-Pen$^2$, D-Pen$^5$-enkephalin: DPDPE) and delta$_2$ (deltorphin II) receptors. DAMGO (1-20 ng) dose-dependently increased tail-flick latencies in the PAG and RVM, and sub-antinociceptive doses of DAMGO administered simultaneously to the two sites produced strong multiplicative interactions. In contrast, neither U50,488H (20 μg) nor DPDPE (20 μg) altered latencies in the PAG and RVM, and failed to produce any interactive effects when paired with DAMGO. Deltorphin (20 μg) produced a mild antinociception in the PAG and RVM, and its simultaneous administration to the two sites produced small interactive effects. Simultaneous administration of deltorphin (10 μg) and DAMGO (3 ng) to the two sites produced a profound multiplicative interaction. In contrast, deltorphin and DPDPE failed to produce interactive effects. These data indicate that antinociceptive opioid synergy occurs between the PAG, RVM and DLP with the most pronounced effects in the PAG and RVM mediated by mu$_1$, and to a lesser degree, delta$_2$, opioid receptors.

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This dissertation would not have been possible without the unselfish contributions of my mentor, Dr. Richard Bodnar. I will always be grateful to him for his boundless enthusiasm, tireless guidance and uncanny insight into neuroscientific research. Thanks again for your time, patience and for taking a chance on me.

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## Glossary of Abbreviated Terms

1. PAG = periaqueductal gray.
2. DLP = dorsal lateral pons.
3. RVM = rostral ventral medulla.
4. NRM = nucleus raphe magnus.
5. NRGC = nucleus reticularis gigantocellularis.
6. LC = locus coeruleus.
7. DLF= dorsal lateral funiculus.
8. DAMGO = D-Ala$^2$,met-phe$^4$,gly(ol)$^5$-enkephalin; mu agonist.
9. 5-HT = Serotonin.
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. Naz = naloxonazine; mu$_1$ antagonist.
14. ß-FNA = beta-funaltrexamine; mu antagonist.
15. Nor-BNI = nor-binaltorphamine; kappa antagonist.
16. DALCE = D-Ala$^2$,Leu$^5$,Cys$^6$-enkephalin; delta antagonist.
17. DADLE =[$^3$H]D-Ser$^2$,Leu$^5$,Thr$^6$-enkephalin; general delta agonist.
18. DSLET =[$^3$H]D-Ala$^2$,D-Leu$^5$-enkephalin; general delta agonist. 19.ICS205930 = serotonin antagonist.
20. ICI174864 = general delta antagonist.
21. NTI= naltrindole; delta selective, non-peptide antagonist.
22. TRIMU-5 = mu$_2$ agonist/mu$_1$ antagonist.
23. POMC = pro-opiomelanocortin.
24. ACTH = adrenocorticotropin.
25. AMSH = alpha-melanocyte-stimulating hormone.
26. ß-LPH = beta lipotropin.
27. gamma-MSH = gamma-melanocyte-stimulating hormone.
28. EKC = ethylketocyclazocine.
29. MPE = Maximal Percentage Effect.
30. ED = effective dose.
31. ng = nanogram.
32. µg = microgram.
33. i.t. = intrathecal.
34. i.c.v. = intracerebroventricular.
35. i.c. = intracerebral.
36. IP = intraperitoneal.
37. IM = intramuscular.
38. IV = intravenous.

# INTRODUCTION

## Statement of Aims:

A pivotal issue contributing to the understanding of the modulation of pain perception was the discovery of endogenous pain-inhibitory systems producing centrifugal opioid inhibition of nociceptive input at both supraspinal and spinal levels of the neuraxis. Since the discoveries of the endogenous opioid peptide families and multiple opioid receptor subtypes, much research has focused on the characterization and activation of intrinsic pain-inhibitory systems. The delineation of the spinal and supraspinal systems was characterized by the central analgesic properties of morphine or electrical stimulation delivered to specific supraspinal sites (see reviews: Mayer and Price, 1976; Yaksh and Rudy, 1978) and the ability of opiates to produce antinociception following intrathecal administration to the spinal cord (see reviews: Yaksh, 1984 a,b). The relationship of individual supraspinal loci with respect to other supraspinal and spinal loci implicated in pain inhibition has been the subject of neuroanatomical, neurophysiological and neurochemical analyses (see reviews: Basbaum and Fields, 1984; Fields and Basbaum, 1978; Fields, Heinricher and Mason, 1991; Gebhart, 1982). One means of analyzing whether interactive relationships between opiate-sensitive analgesic sites exist is the study of synergy. Synergy was operationally defined as a greater than additive

effect and was chosen over the comparable term, multiplicative, to be consistent with statistical analyses. Additionally, the term, potentiation which invariably refers to an increase in effect, is not used synonomously with synergy nor multiplicative effects. Yeung and Rudy (1980a) initially demonstrated that simultaneous administration of sub-analgesic dose of opiates to spinal and supraspinal sites produces synergistic antinociceptive interactions. However, little work has been accomplished investigating the existence of multiplicative antinociceptive interactions between specific opiate-sensitive supraspinal sites, and whether synergy between two supraspinal sites occurs. Microinjection mapping studies have identified mesencephalic, metencephalic and mylencephalic regions involved in the mediation of opiate analgesia, including the periaqueductal gray (PAG), locus coeruleus (LC), nucleus raphe magnus (NRM) and the nucleus reticularis gigantocellularis (NRGC) (Bodnar, Williams, Lee, and Pasternak, 1988; Fang, Fields, and Lee, 1986; Jensen and Yaksh, 1986a,b; Schmauss, Shimohigashi, Jensen, Rodbard, and Yaksh, 1985; Schmauss and Yaksh, 1984; Yaksh and Rudy, 1978).

Endogenous opiate pain-inhibition has been proposed to originate in the mesencephalic PAG and adjacent dorsal raphe nucleus, project to the NRM and NRGC, and send descending projections through the dorsolateral funiculus to the dorsal

horn of the spinal cord (Basbaum and Fields, 1984; Bowker and Abbott, 1990). Fields and Basbaum (1978) initially described this supraspinal pathway as an excitatory link from the PAG to the medullary NRM, and an inhibitory link from the NRM to the substantia gelatinosa of the spinal cord. Additionally, serotonergic synapses ($5HT_2$ and $5HT_3$) in the ventral medial medulla participate in the mediation of mesencephalic morphine antinociception (Kiefel, Cooper, and Bodnar, 1991, 1992). Electrophysiological studies have implicated activation of the NRM and NRGC in the mediation of opiate analgesia elicited from the PAG (Fields et al., 1991; Fields and Basbaum, 1978; Gebhart, 1982). Basbaum and Fields (1984) subsequently proposed a role for the NRGC, the LC, and spinally-projecting noradrenergic pathways as being important in this pain-inhibitory system. The PAG sends descending projections to a number of brainstem nuclei, including the dorsal lateral pons (DLP), NRM, NRGC, nuclei raphe pallidus, reticularis magnocellularis gigantocellularis, and paragigantocellularis lateralis and ventralis, which in turn project to the spinal cord (Advokat, 1988; Beitz, Mullett and Weiner, 1983; Mantyh, 1983). Further, reciprocal connections between the LC and the rostro-ventral medulla (RVM) also exist (Chance, 1980; Clark and Proudfit, 1991; Ennis and Aston-Jones, 1986, 1987; Moore and Bloom, 1979) with the former contributing spinal noradrenergic projections to the dorsal horn (Ennis and

Aston-Jones, 1987; ; Moore and Bloom, 1979; Nygren and Olson, 1977; Nygren, Olson and Seiger, 1977; Olson and Fuxe, 1971, 1972).

Given that antinociceptive synergy occurs between supraspinal and spinal opiate-sensitive sites, and given that anatomical and physiological connections exist between these supraspinal sites, it is the central premise of this dissertation that antinociceptive synergy will occur following opiate microinjections into pairs of the following sites: PAG/RVM, DLP/RVM and PAG/DLP.

**The specific aims are as follows:**

1. To provide detailed dose-response curves for morphine antinociception at the following supraspinal sites: the PAG, DLP, and RVM. This was necessary to choose the appropriate doses for additional studies involving synergy and to obtain $ED_{50}$ values for each site. Following the determination of morphine antinociception, sub-analgesic doses of morphine were chosen and administered simultaneously to pairs of the above-mentioned sites. Since synergy occurred between pairs of these sites, dose response curves were determined, whereby a given dose was held constant at the first site and variable doses were tested at the second site, and vice-versa. Since the $mu_1$ receptor has been implicated in opiate antinociception elicited from each of these sites individually (Bodnar et al., 1988; Bodnar, Paul, and Pasternak, 1991), multiplicative interactions between sites

were evaluated in the presence or absence of pretreatment with the selective $mu_1$ antagonist, naloxonazine. This allowed one to ascertain the opiate receptor subtype modulating the synergistic interaction between opioid pain inhibitory sites.

2. At the completion of the first experiment in which morphine was solely utilized, additional opioid agonists were tested. Selective opioid receptor subtype agonists for the mu (D-Ala$^2$, met-Phe$^4$, Gly (ol) $^5$-enkephalin: DAMGO), kappa (U50488H) and $delta_1$ (DPDPE) receptors were respectively examined to determine the extent of antinociception in the two following sites: the PAG and the RVM. Then these agonists were evaluated at specific sub-antinociceptive doses in the PAG and RVM to determine whether they produced multiplicative interactions.

3. Finally, dose-response curves for deltorphin antinociception in the PAG and RVM were performed. Since deltorphin has been postulated to be a $delta_2$ receptor agonist with some mu characteristics (Quirion, Zajac, Morgat and Roques, 1983), additional synergistic studies with deltorphin were performed. Sub-antinociceptive doses of deltorphin were simultaneously administered intracerebrally into the PAG and RVM as described previously.

To provide the underlying conceptual basis, background, and rationale for these aims, the Introduction will examine: a) the classification of endogenous opioid peptide families,

b) multiple opioid receptor subtypes, including selective agonists and antagonists, c) the organization of the endogenous opioid pain control system, including supraspinal and spinal antinociception d) the physiology of the opioid pain inhibitory system, e) the pharmacology of the opioid pain inhibitory system, f) the functional interrelationship between opioid systems in the neuraxis, and g) a rationale for the present experiments.

## A. Endogenous Opioid Peptide Families.

Opioid Peptides: It was well known in the early 1970's that opiates modulate pain perception at the supraspinal levels of the neuraxis (Jacquet and Lajtha, 1973, 1974; Pert and Yaksh, 1974). In 1975, Hughes and his colleagues reported the structure of two opioid pentapeptides, methionine-enkephalin (H-Tyr-Gly-Gly-Phe-Met-OH) and leucine-enkephalin (H-Tyr-Gly-Gly-Phe-Leu-OH), from pig and cow brain (Hughes, 1975a,b). Both peptides had potent agonist activity at opiate receptor sites producing a dose-related inhibition of electrically-evoked contractions of the guinea-pig ileum (Creese and Snyder, 1975) and the mouse vas deferens (Hughes, Kosterlitz and Leslie, 1975). The amino acid sequence of met-enkephalin was contained in positions 61-65 of Beta-lipotropin (B-LPH), a 91 amino-acid peptide isolated from the anterior lobe of the pituitary of sheep, pig and man (Cox, Opheim, Teschemacher and Goldstein,

1975). Beta-endorphin (61-91), the C-terminal fragment of B-LPH also possessed potent opioid activity in vitro and in vivo assays (Cox et al., 1975). Two other opiate-like peptides, alpha-endorphin (B-LPH:61-76) and gamma-endorphin (B-LPH: 61-77) were isolated from the hypothalamus and posterior pituitary as well (Guillemin, Ling and Burgus, 1976).

Earlier investigators (Hughes, 1975a; Simon, 1975; Simon, Hiller and Edelman, 1973; Terenius, 1973; Terenius and Wahlstrom, 1975) identified that opioid peptides are characterized by a common core amino acid sequence of Tyr-Gly-Gly-Phe-Met or Leu (yielding met- and leu-enkephalin, respectively). However, three distinct precursor molecules or prohormones, corresponding to the three distinct genes were subsequently characterized and were differentially distributed in the central nervous system (Akil, Watson, Young, Lewis, Khachaturian, and Walker, 1984; Basbaum and Fields, 1984; Burkhardt, Frederickson, and Pasternak, 1982; Eipper and Mains, 1980; Guillemin, Ling and Burgus, 1976; Herz, 1987; Kimura, Lewis, Stern, Rossier, Stein, and Undenfriend, 1980). Each of the known endogenous opioid ligands originates from one of the following three precursors: i) the beta endorphin/ACTH or POMC precursor, ii) the pro-enkephalin precursor, ii) and the pro-dynorphin/neo-endorphin precursor.

**i) Beta-endorphin.** Pro-opiomelanocortin (POMC) is the

common precursor for beta-endorphin, adrenocorticotropic hormone (ACTH), alpha-melanocyte-stimulating hormone (A-MSH), beta-lipotropin (beta-LPH) and related bioactive peptides (Eipper and Mains, 1978; Mains, Eipper, and Ling, 1977). Beta-endorphin, the 31-residue C-terminal component of B-LPH, is generated from the post-translational process of B-LPH from pro-opiomelanocortin (Guillemin et al., 1976; Hollt, Seizinger, Garzon, Loh, 1983). Although the met-enkephalin opiate core is contained within beta-endorphin, post-translational processing of beta-endorphin does not yield a met-enkephalin end product. $ACTH_{1-39}$ is further cleaved into alpha-melanocyte-stimulating hormone (alpha-MSH) and corticotropin-like intermediate lobe peptide (CLIP) (Brownstein, 1980; Eipper and Mains, 1978; Mains, Eipper and Ling, 1977). The amino terminus of the POMC gene also contains an active ACTH/MSH core, namely gamma-melanocyte-stimulating hormone (gamma-MSH) (Eipper and Mains, 1980; Guillemin, 1976).

Within the central nervous system, beta-endorphin-containing cells are found in high concentrations in the arcuate nucleus/peri-arcuate nucleus of the medial-basal hypothalamus (Bloom, Battenberg, Rossier, Ling, Leppaluoto, Vargo and Guillemin, 1977; Watson, Akil, Richard and Barchas, 1978; Watson, Barchas, and Li, 1977; Watson, Richard, and Barchas, 1978), medulla (Joseph, Pilcher, and Bennett-Clark, 1983) and the anterior and intermediate lobes

of the pituitary (Bloch, Bugnon, Fellman and Lenys, 1978; Brownstein, 1980). Beta-endorphin-containing neurons in the arcuate nucleus have short, local projections to other hypothalamic structures including the suprachiasmatic and paraventricular nuclei and the median eminence, and long-projecting pathways innervating limbic structures, including the amygdala, septum and bed nucleus of the stria terminalis (Guillemin et al., 1976; Kangawa, Minamino, Chino, Sakakibara and Matsua, 1981; Watson et al., 1977, 1978). Hypothalamic beta-endorphin projections also terminate in the caudal part of the nucleus tractus solitarius (NTS) which projects laterally to the lateral reticular nucleus (Khachaturian, Lewis, Schafer and Watson, 1985). POMC cells in the arcuate nucleus project rostrally through periventricular diencephalic and telencephalic areas, innervating the preoptic area, the amygdala, septum and the bed nucleus of stria terminalis. Arcuate POMC neurons project laterally through the medial-basal hypothalamic region and the temporal cortex. Caudally-projecting POMC fibers innervate the periventricular thalamus, the PAG, dorsal lateral pons, nucleus tractus solitarius, parabrachialis, ambiguus, the dorsal motor nucleus of the vagus nerve (Guillemin et al., 1976; Khachaturian et al., 1985) and such medullary areas as the NRM, NRGC and reticularis lateralis (Khachaturian, Watson, Lewis, Coy and Goldstein, 1982).

**ii) Pro-enkephalin.** In addition to B-LPH, other larger, molecular weight peptides containing the met- and leu-enkephalin sequences have been isolated. Within the pro-enkephalin precursor are several active opioid peptides, including leu-enkephalin, met-enkephalin, met-enkephalin-Arg-Phe and met-enkephalin-Arg-Gly-Leu (Comb, Herbert and Crea, 1982; Kimura et al., 1980). Pro-enkephalin, a 50,000 molecular weight precursor protein molecule was isolated from the adrenal glands and contains met-enkephalin and leu-enkephalin sequences at a 7:1 ratio (Calcagnetti, 1983; Calcagnetti, Faneslow, Helmstetter, and Bowen, 1989; Hollt, Tulnay, Woo, Loh and Herz, 1982; Hughes 1975b; Kimura et al., 1980; Lewis, Khachaturian and Watson, 1985;). Pro-enkephalin contains four copies of met-enkephalin, and one of each of leu-enkephalin, met-enkephalin-Arg6-Phe7, and met-enkephalin-Arg6-Gly7-Leu8. Whereas the only source of met-enkephalin is pro-enkephalin, leu-enkephalin may be cleaved from pro-dynorphin as well. Enkephalinergic perikarya are found in the telencephalon, diencephalon, mesencephalon, metencephalon, mylencephalon and the dorsal horn of the spinal cord (Hökfelt, Elde, Johansson, Terenuis and Stein, 1977; Khachaturian, Lewis, Hollt and Watson, 1983; Sar, Stumpf, Miller, Chang and Cuatrecasas, 1978). Met- and leu-enkephalin are also present in all pituitary lobes in which larger pro-enkephalin-derived peptides are found in trace amounts (Pert and Snyder, 1973 a,b; Simon,

1975; Wolozin and Pasternak, 1981). Neural pathways containing pro-enkephalin are widely distributed throughout the central and peripheral nervous systems (Hökfelt, et al., 1977; Terenius and Wahlstrom, 1975). Enkephalinergic neurons are found along most of the neuraxis, in areas such as the cerebral cortex, olfactory tubercle, amygdala, hippocampus, bed nucleus of the stria terminalis, and preoptic area. In the diencephalon, enkephalinergic cell bodies are found in most hypothalamic nuclei, as well as the periventricular and lateral geniculate nuclei of the thalamus. Mesencephalic enkephalin-containing structures include the colliculi, PAG, and the interpeduncular nucleus. In the pons and the medulla, perikarya are found in the raphe nuclei, NRGC, nucleus reticularis paragigantocellularis, nucleus tractus solitarius, the lateral reticular nuclei, spinal trigeminal nucleus (Khachaturian et al., 1982). Virtually all enkephalinergic neurons have very short axonal projections, and typically act as interneurons in cell groups where they reside.

**iii) Prodynorphin.** Prodynorphin was isolated from brainstem, hypothalamic, posterior pituitary and gut extracts (Goldstein, 1984; Goldstein and James, 1984; Goldstein, Tachibana, Lowney, Hunkapiller and Hood, 1979; Kangawa et al., 1981). The prodynorphin precursor is cleaved to produce three leu-enkephalin-containing peptides: alpha and beta-neo-endorphin, dynorphin A and dynorphin B

(Goldstein, Fischli, Lowney, Hunkapiller and Hood, 1981; Kangawa et al., 1981). The three peptides derived from this prohormone include dynorphin $A_{1-17}$, dynorphin $A_{1-8}$ (DYN B), and alpha- and beta-neoendorphin with all having a N-terminal leu-enkephalin sequence (Hollt, 1986; Hollt, Przewlocki, Haarman, Almeida, Kley, Millan and Herz, 1986). Immunoreactive dynorphin perikarya are distributed in several cerebral cortical areas, striatum, amygdala, and hippocampus (Goldstein et al., 1981; Seizinger, Hollt and Herz, 1981; Suda, Tozawa, Tachibana, Demura and Shizume, 1982; Tulunay, Jen, Chang, Loh and Lee, 1981). Brain dynorphin-related products are also found in hypothalamus (suprachiasmatic, paraventricular, supraoptic, and arcuate nuclei), PAG, brainstem (spinal trigeminal nucleus, nucleus tractus solitarius, lateral reticular nucleus) and the dorsal horn of the spinal cord. Comparisons of endorphin, enkephalin and dynorphin terminals in the PAG reveal that the latter are located more ventrally (Pert, Snowman, and Snyder, 1974). Whereas immunoreactive enkephalin is densely concentrated in laminae I, II and V, dynorphin's spinal distribution is limited to the marginal (Lamina I) zone (Glazer and Basbaum, 1981).

**B. <u>Opiate Receptor Subtypes</u>.**

Based upon the initial studies demonstrating sites sensitive to morphine analgesia (Atweh and Kuhar, 1977 a,b;

Herz, Albus, Metys, Schubert, and Teschemacher, 1970; Jacquet and Lajtha, 1973, 1974; Pert and Yaksh, 1974; Sharpe, Garnett, Cicero, 1974; Tsou and Jang, 1964; Yaksh, Yeung and Rudy, 1976; Tyers, 1981), interrelated mesencephalic, metencephalic and mylencephalic structures were described at which opiates modulate the perception of noxious stimuli in humans and animals by activating an endogenous opioid system (Akil, et al., 1984; Basbaum and Fields, 1984; Fields and Basbaum, 1978; Yaksh and Rudy, 1978). Following the discovery of the opiate receptor in 1973 (Pert and Snyder, 1973 a,b; Simon et al., 1973; Terenius, 1973), pharmacological, bioassay and biochemical approaches indicated the existence of multiple opiate receptor subtypes: mu, delta, kappa, sigma and epsilon (e.g., Goodman and Snyder, 1982; Goodman, Snyder, Kuhar and Young, 1980; Mansour, Khachaturian, Lewis, Akil and Watson, 1987, 1988; Lord, Waterfield, Hughes, and Kosterlitz, 1977; Martin, Eades, Thompson, Huppler, and Gilbert, 1976; Schulz, Faase, Wuster, and Herz, 1979; Ward and Takemori, 1983). However only three of these distinct subtypes were proposed to explain the different patterns of the opioid agonists: mu (morphine), kappa (ketocyclazocine) and sigma (SKF 10,047). The latter subtype was subsequently not considered an opioid receptor because it was unaffected by the opiate antagonist, naloxone (Vaupel, 1983; Zukin, Brady, Slifer and Balster, 1984). Using bioassay and binding studies, Lord and

colleagues (1977) found dissociations between morphine and enkephalin peptides, and suggested the existence of a delta opioid receptor.

In an attempt to clarify the receptor mechanisms mediating spinal and supraspinal opiate analgesia, the selectivity of gene-related opioid peptides was correlated with opiate receptor subtypes (Chang and Cuatrecasas, 1979). Based on the differential pattern of binding affinities between receptors and opioid families, delta receptors were associated with pro-enkephalin-derived opioid peptides (Bowen, Hellewell, Kelemen, Huey and Stewart, 1987; Cotton, Giles, Miller, Shaw and Timms, 1984), kappa receptors were associated with pro-dynorphin-derived opioid peptides and mu-epsilon receptors were associated with POMC-derived opioid peptides. Subsequent research established that considerable cross-reactivity occurs between gene-related families and receptor subtypes. Another problem with this proposition is the inconsistent localization between a given receptor and its preferred substrate. Opioid receptor subtypes are heterogeneously distributed throughout the neuraxis. Moreover, the anatomy of opioid receptors and peptides in the medulla and spinal cord generally conform to expectations (Goodman, Snyder, Kuhar and Young, 1980; Mansour, Khachaturian, Lewis, Akil and Watson, 1986). However, elsewhere in the brain, and especially in the forebrain, very little ligand-receptor relationship is

observed (Herkenham, 1987). For example, mu and delta receptors are strikingly sparse in the areas that contain the densest levels of peptides, namely the medial hypothalamus and central amygdaloid nucleus. Conversly, mu receptors are densest in thalamic nuclei that contain no visible mu-like immunoreactivity (Burkhardt et al., 1982; Herkenham, 1987).

Studies employing selective opioid receptor subtype agonists and antagonists have attempted to define the receptor types involved in the mediation of supraspinal and spinal opioid antinociception. Supraspinal administration of either mu (DAMGO) and delta (D-Ser$^2$,Leu$^5$-enkephalin-Thr$^6$ (DSLET), D-Ala$^2$,D-Leu$^5$-enkephalin (DADL), D-Pen$^2$, D-Pen$^5$-enkephalin (DPDPE)) receptor agonists were found to be active in several antinociceptive assays (Heyman, Koslo, Mosberg, Tallarida and Porreca, 1986; Heyman, Mulvaney, Mosberg, Porreca, 1987; Howe and Yaksh, 1982; Jensen and Yaksh, 1986 a,b,c; Jiang, Bowen, Mosberg, Rothman and Porreca, 1990; Pasternak, 1980; Porreca, Heyman, Mosberg, Omnas, and Vaught, 1987; Porreca, Mosberg, Hurst, Hruby, and Burks, 1984; Schmauss and Yaksh, 1984; Takagi, Satoh, Akaike, Shibata, and Kuraishi, 1978).

i) Mu receptors: The mu receptor has been sub-characterized into $mu_1$ and $mu_2$ subtypes based upon pharmacological and biochemical assays (see review: Pasternak and Wood, 1986). The $mu_1$ binding site is a common

high affinity site, binding morphine, ethylketocyclazocine, enkephalin peptides and analogues and beta-endorphin with equally high affinity. The $mu_2$ binding site binds morphine with high affinity and exhibits low affinity for other opioid peptides.

Mu receptors are widely distributed throughout the forebrain, midbrain and hindbrain. The highest densities of mu receptors are found in the neocortex, caudate-putamen, nucleus accumbens, thalamus, hippocampus, amygdala, inferior and superior colliculi, NTS, the spinal trigeminal nucleus and the dorsal horn of the spinal cord. Moderate densities of mu receptors are found in the PAG and raphe nuclei, including the NRM. Relatively little binding is seen in the hypothalamus, preoptic area and globus pallidus (Mansour, et al., 1988). In assessing pharmacological effects of the mu receptor, selective agonists (DAMGO: Handa, Lane, Lord, Morgan, Rance and Smith, 1981; Besse, Lombard and Besson, 1992), and antagonists (beta-funaltrexamine, ß-FNA: Portoghese, Larson, Sayre, Fries and Takemori, 1980; Takemori, Larson and Portoghese, 1981) have been developed.

ii) <u>$Mu_1$ receptors</u>: The $mu_1$ binding site is a common high affinity site, binding morphine, ethylketocyclazonine, enkephalin peptides and beta-endorphin with equally high affinity. However, the $mu_2$ site selectively binds morphine-like compounds more potently than enkephalins (Clark,

Houghten and Pasternak, 1988; Pasternak and Wood, 1986; Wolozin and Pasternak, 1981). Autoradiographic studies demonstrate that $mu_1$ and $mu_2$ binding sites have similar, though not identical distributions (Goodman and Pasternak, 1985; Moskowitz and Goodman, 1985 a,b). $Mu_1$ binding is higher in the frontal cortex, striatum, ventral pallidum, nucleus accumbens, medial thalamus, interpeduncular nucleus, median raphe and PAG. Naloxonazine, an irreversible $mu_1$ antagonist (Hahn, Carroll-Buatti and Pasternak, 1982) has been used pharmacologically to discern $mu_1$ actions. By using the $\mu_1$-selective antagonist naloxonazine (Hahn et al., 1982) and the differential selectivity of a series of opioid peptides, opiate antinociception elicited from the PAG, DLP, NRM and NRGC can be eliminated by naloxonazine, implicating the $mu_1$ receptor subtype in these effects (Bodnar et al., 1988).

iii) Delta receptors: Delta receptors are most dense in the olfactory bulb, neocortex, striatum, accumbens and amygdala with little binding observed in the thalamus, hypothalamus and brainstem (Gacel, Zajac, Delay-Goyet, Dauge and Roques, 1988). Pharmacological analysis of the delta receptor has utilized the general delta agonists, DSLET and DADL (Mosberg, Hurst, Hruby, Gee, Yamamura, Galligan and Burks, 1983a) and the general delta antagonist, ICI,174864 (Cotton et al., 1984). Recent development of selective delta agonists and antagonists indicate the existence of

$delta_1$ and $delta_2$ subtypes (Negri, Potenza, Corsi, and Melchirri, 1991). The $delta_1$ receptor subtype has been characterized by the agonist actions of DPDPE (Mosberg, Hurst, Gee, Yamamura, Galligan, and Burks, 1983b) and the long-term antagonist actions of D-$Ala^2$, $Leu^5$, $Cys^6$-enkephalin (DALCE: Bowen et al., 1987; Jiang Takemori, Sultana, Portoghese, Bowen, Mosberg and Porreca, 1991). The $delta_2$ receptor subtype has been characterized by the agonist actions of D-$Ala^2$-deltorphin II (Jiang, Heyman, Sheldon, Koslo and Porreca, 1990) and the antagonist actions of naltrindole (Portoghese, Sultana, Nagase and Takemori, 1988; Portoghese, Sultana and Takemori, 1988; Sofuoglu, Portoghese and Takemori, 1991). Indeed, the effects of $delta_1$ and $delta_2$ agonists and antagonists have been dissociated from each other in antinociceptive assays (Mattia, Vanderah, Mosberg and Porreca, 1991; Jiang et al., 1991).

Whereas $mu_1$ receptors are implicated in supraspinal antinociception (Bodnar et al., 1988; Pasternak and Wood, 1986), delta and kappa receptors have been implicated in spinally-mediated antinociception. Administration of selective delta and kappa agonists and antagonists indicate their respective potencies in the spinal cord (e.g., Yaksh, 1984a,b; Porecca et al., 1984; Porreca, 1987; Wuster, Schulz and Herz, 1980; Heyman et al., 1987, 1988). Evidence implicating the mu receptor in spinal opiate antinociception has involved the $mu_2$ binding site in these effects (Heyman,

Vaught, Raffaand Porreca, 1988; Paul, Bodnar, Gistrak, and Pasternak, 1989). In these studies, intrathecal pretreatment with the irreversible mu antagonist, ß-FNA (Takemori, Larson and Portoghese, 1981), but not naloxonazine, eliminated spinal analgesia induced by morphine and the mu agonist, DAMGO.

iv) Kappa receptors: The distribution of kappa opioid receptors includes dense binding in the striatum, accumbens, amygdala, hypothalamus, neural lobe of the pituitary, the median eminence and NTS and moderate binding in the PAG, NRM, other raphe nuclei, spinal trigeminal nucleus and the dorsal horn of the spinal cord (Mansour et al., 1988). Whereas the prototypical kappa agonist is U50,488H (VanVoigtlander, Lahti and Ludens, 1983; Ho and Takemori, 1990), nor-binaltorphamine (NOR-BNI) is a selective antagonist (Portoghese, Lipkowski and Takemori, 1987; Takemori, Ho, Naeseth and Portoghese, 1988). The kappa receptor has been recently subclassified into $K_1$, $K_2$, and $K_3$ binding sites (Zukin, Eghbali, Olive, Unterwald and Tempel, 1988; Rothman, Bykov, deCosta, Jacobson, Rice and Brady, 1990). These data indicate that U50,488H and Nor-BNI are respective $k_1$ agonists and antagonists, and the $K_3$ site has been identified using naloxone benzolhydrazone (NalBzoH) (Clark, Liu, Price, Hersh, Edelson and Pasternak, 1989; Gistrak, Paul, Hahn and Pasternak, 1989; Paul, Levison, Howard, Pack, Hahn and Pasternak, 1990).

Supraspinal administration of kappa receptor ligands (dynorphin-related peptides) fail to elicit antinociception (Friedman, Jen, Chang, Lee, and Loh, 1981; Chavkin, James, and Goldstein, 1982), and supraspinal administration of kappa-selective analogues (e.g., U50,488H) produce a minor antinociceptive effect (Millan, 1989, 1990). Indeed, the prototypical kappa agonist, ethylketocyclazocine, fails to produce intracerebral supraspinal antinociception when administered into the PAG and LC alone, but produces antinociception when administered simultaneously into the two sites (Bodnar et al., 1991). The resultant antinociception is eliminated by naloxonazine pretreatment, indicating that ethylketocyclazocine acts as a partial $mu_1$ agonist.

**C. <u>Organization of the Endogenous Pain Control System.</u>**

Supraspinal opioid antinociception appears to be modulated by neurons which originate in the midbrain PAG, synapse in the medullary NRM, NRGC, and NRGC pars alpha (collectively known as the rotro-ventral medulla: RVM), and project to the substantia gelatinosa of the spinal cord through the dorsolateral funiculus (Basbaum and Fields, 1984; Fields and Basbaum, 1978; Mantyh and Peschanski, 1982). This model of descending pain inhibition is supported by various lines of research. Lesions placed in either the NRM (Proudfit and Anderson, 1975) or the dorsolateral funiculus (Kitahata, Yosaka, Taub, Bonikos and

dorsolateral funiculus (Kitahata, Yosaka, Taub, Bonikos and Hoffert, 1974; LeBars, Menetrey, Conseiller and Besson, 1975; Murphin, Bennett and Mayer, 1976) attenuate antinociception elicited by either electrical stimulation or morphine microinjection into the PAG. Both of these forms of antinociception in the PAG excite RVM neurons (Behbehani and Zemlan, 1986; Lovick, West and Wolstencroft, 1978; Pomeroy and Behbehani, 1979; Mohrland and Gebhart, 1980a) and inhibit nociceptive-sensitive dorsal horn neurons (Mayer and Liebeskind, 1974; Gray and Dostrovsky, 1983). The following sections will review in detail: i.) an anatomical analysis of the periaqueductal gray, ii.) the medulla and, iii.) the locus coeruleus of the dorsal lateral pons.

**Supraspinal System: i) Periaqueductal Gray.** The PAG area surrounding the cerebral aqueduct throughout the midbrain, is a cytoarchitectonically complex region that consists of rather densely-packed, small cells difficult to subdivide anatomically (Gioia, Bianchi and Tredici, 1984; Hamilton, 1973). Recent anatomical tracing studies have compartmentalized the PAG into functionally-distinct units (Beitz, Shepard and Wells, 1983; Van Bockstaele, Aston-Jones, Pieribone, Ennis and Shipley, 1991). Based upon physiological stimulation studies (e,g., Mayer and Price, 1976), the PAG is a heterogeneous region with functions including pain modulation, as well as sensory integration and autonomic regulation. The PAG has reciprocal

locus coeruleus and medullary nuclei raphe magnus and reticularis gigantocellularis. Anterograde tracing studies have revealed projections to numerous forebrain sites (Van Bockstaele et al., 1991), the superior colliculus (Mitchell, Dean and Redgrave, 1988), the cuneiform nucleus (Redgrave, Dean, Mitchell, Odekunle and Clark, 1988), the NRM (Beitz, 1982a), the nucleus paragigantocellularis (Beitz, 1982b), the nucleus ambiguus, the nucleus of the solitary tract (Bandler and Tork, 1987), and the NRGC (Van Bockstaele, Pieribone and Aston-Jones, 1989). In turn, brainstem inputs to the PAG include the nucleus cuneiformis (Edwards, 1975), the pontine reticular formation, and the LC. Taken together with the known direct spinal input to the PAG, the former two regions may provide a possible feedback relay regulating nociceptive input that activates PAG neurons in the dorsolateral, ventrolateral and dorsomedial subregions (Beitz et al., 1983; Shipley, McLean and Behbehani, 1987; Smith, Perotti, Crisp, Cabral, Long and Scalzitti, 1988). Small amounts of morphine or endogenous opioids injected directly into various regions of the PAG of rats, cats, and monkeys produces strong antinociceptive effects (Malick and Goldstein, 1977; Pert and Yaksh, 1974; Tsuo and Jang, 1964; Yaksh and Rudy, 1978). Antinociception is known to be effective following stimulation or opiate microinjection into the ventrolateral region of the PAG (see reviews: Bausbaum and Fields, 1984; Akil et al., 1984; Proudfit,

1988; Sharpe, Garnette and Cicero, 1974). The caudal and ventrolateral regions of the PAG contain a dense innervation of enkephalin cells and terminals. This distribution shifts dorsally as one moves rostral in the midbrain (Beitz, 1982c, 1985). Dynorphin cells are concentrated just ventral to the aqueduct, along the rostral caudal extent of the PAG (Burnett and Gebhart, 1991). POMC-positive terminals are distributed within the ventral and ventro-lateral portions of the PAG, including the dorsal raphe nucleus (Khachaturian et al., 1982). Opiate antinociception in the PAG was reversed by the opiate receptor antagonist naloxone (Bodnar, Kelly, Spiaggia, Ehrenberg and Glusman, 1978; Chesler, 1977).

**ii) Medulla.** An area of major importance in the study of the organization of the opiate system is the rostro-ventral medulla (RVM). Two principal distributions of sites modulating opioid analgesia are as follows: a) the paramedial medulla which includes the NRGC, and b) the medial medulla which includes the NRM and NRGC, pars alpha. Also included in the RVM is the nucleus reticularis magnocellularis, the nucleus paragigantocellularis and the lateral reticular nucleus. Efferent PAG-RVM and RVM-dorsal horn projections have been identified using anatomical and physiological approaches (Guyenet and Young, 1987; Yeung and Rudy, 1980a; Yaksh, 1979; Proudfit and Anderson, 1975; Miyamoto, Morita, Kitabata, Yamanishi, Kishioka, Ozaki and

Yamamoto, 1991). The modulatory action of this pathway upon nociceptive processes involves neurotransmitters and peptides intrinsic to the RVM, including 5-HT (Clements et al., 1985), norepinephrine (NE) (Moore and Bloom, 1979), GABA (Clements, Madl, Johnson, Larson and Beitz, 1987), enkephalin, neurotensin and excitatory amino acids (Beitz, 1982b, 1983).

Initial studies by Takagi and colleagues (1977) indicated that the RVM, and specifically the NRGC could sustain morphine analgesia following microinjections. Subsequent mapping studies indicated that antinociception can also be elicited following microinjections of morphine into either the NRM, NRGC and nuclei reticularis paragigantocellularis and reticularis paragigantocellularis lateralis of the RVM (Akaike, Shibata, Satoh and Takagi, 1978; Azami, Llewelyn and Roberts, 1982; Dickenson, Oliveras and Besson, 1979; Levy and Proudfit, 1979; Vasko, Pang and Vogt, 1984). Noradrenergic fibers in the RVM also contribute to localized analgesic responses (Proudfit, 1988; Hammond, Levy and Proudfit, 1980). Although the NRM and NRGC project to several brainstem and spinal cord sites (Gebhart, Sankuhler, Thalhammer and Zimmerman, 1983; Zhuo and Gebhart, 1990 a,b, 1991, 1992), their major descending projections lead to the spinal and trigeminal dorsal horns (Basbaum, Clanton and Fields, 1978). Axons of the NRM and NRGC 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 and VII of the spinal dorsal horn (Holstege, 1988). Additionally, these laminae are known to contain the terminals of small diameter nociceptive primary afferents (Fields and Basbaum, 1978).

The midbrain PAG and adjacent nucleus cuneiformis constitute a major input to the RVM which serves as a relay for midbrain modulatory influences upon spinal nociceptive transmission. Thus, the inhibition of behavioral and dorsal horn neural responses to noxious stimulation produced by electrical or chemical activation of the PAG can be blocked by lesions of or local anesthetic into the rostral medulla (see review: Basbaum and Fields, 1984). It seems clear that morphine, exerting a local action within several brainstem loci, namely the mesencephalic reticular formation, the NRM and the NRGC, can alter nociceptive responses which are organized by both spinal and supraspinal systems (Hentall, Barbaro and Fields, 1991; Chung, Kevetter, Yezierski, Haber, Martin and Willis, 1983).

**iii) Locus Coeruleus.** The NRM and NRGC of the rostral ventral medulla also receive significant input from neurons in the dorsolaterally adjacent pontine tegmentum:(DLP) including noradrenergic projections from the LC (see review: Moore and Bloom, 1979). The DLP supports antinociceptive responses following either electrical stimulation (Segal and

Sandberg, 1977) or morphine microinjection (Bodnar et al., 1988, 1991). Indeed, the major inputs to the LC appear to be quite delimited from the dorsal medulla and the NRGC (Aston-Jones, Ennis, Pieribone, Nickell and Shipley, 1986; Ennis and Aston-Jones, 1986, 1987; Ennis, Shipley, Behbani, Van Bockstaele and Aston-Jones, 1991; Jones and Gebhart, 1986). Clark and Proudfit (1991) propose that part of the spinal noradrenergic contribution to antinociceptive processes is due to activation of a descending projection from the DLP by RVM neurons.

**Spinal System.** Intrathecal administration of opiates and opioid analogues produces a dose-dependent antinociception which can be blocked by naloxone (Yaksh and Rudy, 1978; Yaksh, 1981). This suggests that the antinociception is mediated by the direct action of opiates on spinal cord opioid receptors. The modulation of spinal opioid antinociception has been delineated with the development of selective agonists and antagonists for specific opiate receptor subtypes. Intrathecal administration of mu selective agonists elicits antinociception which is blocked by beta-funaltrexamine, but not naloxonazine, indicating a $mu_2$ mechanism of action (Paul et al., 1989). Delta and kappa agonists produce predominantly spinal antinociception which is blocked by selective antagonists for these receptors (Yaksh, 1984 a,b; Porreca et al., 1984, 1987; Wuster et al., 1980; Heyman et

al., 1987, Heyman, Williams, Burks, Mosberg and Porreca, 1988). Thus, $mu_2$, delta and kappa receptors have been implicated in spinally mediated opioid antinociception.

Intrathecal administration of norepinephrine and serotonin also results in a dose-dependent antinociception which can be attenuated by antagonists for these specific neurotransmitters (Wang, 1977; Yaksh and Wilson, 1979; Reddy, Maderdrut and Yaksh, 1980; Reddy and Yaksh, 1980; Schmauss, Hammond, Ochi and Yaksh, 1983; Howe, Wang and Yaksh, 1983; Fleetwood-Walker, Mitchell, Hope, Moloney and Iggo, 1985; Crisp, Smith, Perrotti and Amedro, 1986; Kellstein, Malseed and Goldstein, 1988; Castiglioni, Gallaway and Coulter, 1978). Studies focusing on the interactions of these neurotransmitter systems and opioids in the mediation of spinal antinociception have produced conflicting results.

Specifically, research regarding opioid modulation of norepinephrine antinociception has demonstrated that intrathecal naloxone has no effect upon intrathecal norepinephrine antinociception (Kellstein et al., 1988) and cross-tolerance is not seen between intrathecal morphine and intrathecal administration of ST-91 ($alpha_2$-adrenergic agonist) (Tung, Yaksh and Wang, 1981). However, other research has shown that cross-tolerance exists between intrathecal morphine and intrathecal norepinephrine or adrenergic agonists (Milne, Cervenko, Jhamandas, Loomis and

Sutak, 1985; Solomon and Gebhart, 1987), and that intrathecal administration of naloxone inhibits the antinociception produced by intrathecal norepinephrine (Loomis, Jhamandas, Milne and Cervenko, 1987). Research focusing on the role of this neurotransmitter in the mediation of opioid antinociception has revealed that neither intrathecal administration of noradrenergic antagonists nor depletion of spinal cord norepinephrine levels affected intrathecal opioid antinociception (Kellstein et al., 1988; Pang and Vasko, 1986).

Studies focusing on opioid mediation of serotonin antinociception has revealed that intrathecal administration of naloxone attenuates intrathecal serotonin antinociception (Kellstein et al., 1988). However, other research revealed a lack of cross-tolerance between intrathecal morphine and intrathecal serotonin (Loomis et al., 1987). Serotonergic modulation of spinal opioid antinociception was documented from the finding that intrathecal serotonergic antagonists attenuate intrathecal morphine antinociception (Kellstein et al., 1988). However, other research showed that depletion of spinal cord serotonin levels by p-chlorophenylalanine and 5,7-dihydroxytryptamine does not attenuate intrathecal morphine antinociception (Yaksh and Rudy, 1978; Vasko et al., 1984).

**D. Physiology of Pain-Inhibitory Circuits. Which neurons modulate nociception in the rostral ventral medulla?**

Electrophysiological studies have further implicated the activation of the rostro-ventral medulla (RVM), which includes the NRM and NRGC, in the mediation of opioid antinociception elicited from the PAG (Lovick et al., 1978; Pomeroy and Behbehani, 1979; Mohrland and Gebhart, 1980; Sandkuhler and Gebhart, 1984 a,b; Zorman, Hentall, Adams and Fields, 1981). Three physiologically-distinct classes of neurons ("on-cells", "off-cells" and "neutral cells") can be identified in the RVM based upon the temporal correlation of changes in their firing with the execution of reflexes elicited by noxious stimulation (Fields, Barbaro and Heinricher, 1988). Recently, Fields and colleagues have introduced a neurophysiological classification system involving cells in the RVM which respond differentially to nociceptive inputs by either increasing ("on-cells") or decreasing ("off-cells") their activity (Fields, Bry, Hentall and Zorman, 1983; Vanegas, Barbaro and Fields, 1984; Barbaro, Heinricher and Fields, 1986; Cheng, Fields and Heinricher, 1986; Fields et al., 1988; Barbaro, Heinricher and Fields, 1989; Fields et al., 1991). Cells of the first class, "on-cells", reliably show a sudden increase in firing just prior to the occurence of a response, which are activated to evoke a withdrawal reflex (Vanegas, Barbaro and Fields, 1984 a,b). "On-cells" are highly active just prior to and during the execution of the tail-flick (D'Amour and Smith, 1941), which is the nociceptive measure used in the

present study. This indicates that "on-cell" firing does not have a potent inhibitory action on nocifensive reflexes. Moreover, administration of systemic morphine or morphine microinjected into the PAG at doses sufficient to block the tail-flick response supresses "on-cell" firing (Barbaro et al., 1986; Cheng et al., 1986; Mason and Fields, 1989).

The second set of cells, "off-cells", cause an abrupt pause in firing prior to the tail-flick response. " Off-cells" therefore, inhibit nociceptive transmission and become continuously active following administration of morphine either systemically or by microinjection into the PAG (Fields et al., 1983; Cheng et al., 1986; Mason and Fields, 1989). The opiate activation of "off-cells" is particularly significant because of the evidence that the modulatory output neurons in the RVM responsible for nocifensor reflex suppression is excited by opiates (Vanegas et al., 1984b).

"On" and "off cells" play a central role in descending nociceptive modulation, and both cell classes are excited by electrical stimulation in the PAG (Fields et al., 1988; Vanegas et al., 1984a). These two physiologically-distinct classes of RVM neurons project to the dorsal horn, where they are likely to exert opposing actions on nociceptive transmission (Fields et al., 1983). Moreover, "on" and "off cells" have also been described in the PAG and the nucleus cuneiformis (Heinricher, Cheng and Fields, 1987; Haws,

Williamson and Fields, 1989), sites which have major projections to the RVM. Therefore, several authors (Fields, et al., 1988; Vanegas et al., 1984b; Mason and Fields, 1989; Bodnar et al.,1988; Van Bockstaele et al., 1991) believe modulation of nociception by the RVM must be interpreted in terms of the interactions of these two populations within the RVM and their terminations in the dorsal horn.

Studies indicate that activation of a GABA-containing input may be responsible for the "off-cell" pause, especially since cell bodies and terminals containing GABA are abundant in the rostral ventral medulla. Consequently, little is known of GABAergic projections to the RVM, however it is believed that very few PAG neurons projecting to the RVM contain GABA. Therefore, opioids may excite "off-cells" causing disinhibition of the cell.

Finally, cells that display no change in firing related to the execution of withdrawal from pain, and are not affected by systemically or centrally administered morphine are called "neutral cells" (Fields et al., 1983; Barbaro et al., 1986). Although neutral cells are present in the RVM, the vast majority of cells have been classified as either on or off-cells. In the basic model of the RVM circuitry, Fields et al., (1988) favored the view that off-cells excite other off cells and inhibit on-cells throughout the RVM, thereby causing antinociception.

**E. <u>Pharmacology of the Pain Control System</u>.**

With the recent development of selective agonists and antagonists for particular receptor subtypes, the involvement of specific opioid receptors in the mediation of supraspinal opioid antinociception has been investigated. This research has demonstrated that the $mu_1$ receptor plays an important role in supraspinal opioid antinociception. Microinjections of morphine, DAMGO (mu) and DSLET (delta, $mu_1$) into the PAG, DLP, NRM and NRGC elicits antinociception that can be blocked by naloxonazine (Bodnar et al., 1988). Some investigations support a supraspinal role for delta receptors (Jensen and Yaksh, 1986a; Porreca et al., 1984, 1987). DPDPE, a $delta_1$-selective agonist produces antinociception following ventricular administration (Porreca et al., 1987). However, DPDPE fails to alter nociceptive responses following microinjection into the PAG, DLP, NRM or NRGC (Bodnar et al., 1988). Kappa receptors do not appear to be participating in supraspinal antinociception (Fang, Haws, Drasner, Williamson and Fields, 1989). Ethylketocyclazocine (EKC), one of the original prototypical kappa agonists produces antinociception following intraventricular administration, but not following administration into either the PAG or DLP (Bodnar et al., 1988). Indeed, EKC pretreatment into these structures interferes with the subsequent development of morphine antinociception. EKC's actions upon morphine antinociception was that of a partial $mu_1$ agonist because

intraventricular EKC antinociception was blocked by naloxonazine and because simultaneous administration of EKC into the PAG and DLP produced a naloxonazine-sensitive antinociception (Bodnar et al., 1991).

Much evidence supports the participation of both serotonergic and noradrenergic systems in the mediation of supraspinal opioid antinociception. As mentioned, the RVM receives input from the DLP (Moore and Bloom, 1979). Clark and Proudfit (1991) have proposed that a spinal noradrenergic contribution to supraspinal antinociception may be due to an activation of a descending projection from the DLP by RVM neurons. Studies focusing on the role of these two neurotransmitters in the mediation of supraspinal antinociception have revealed that systemic or intrathecal administration of serotonergic or noradrenergic antagonists attenuates the antinociception elicited by stimulation of the PAG, or systemic and intracerebral injections of morphine (Yaksh, 1979; Yaksh and Wilson, 1979; Tseng and Tang, 1989; Hammond and Yaksh, 1984; Proudfit and Hammond, 1981). Moreover, intrathecal administration of either serotonergic or noradrenergic antagonists attenuates the antinociception which is elicited from stimulation or microinjection of morphine into the RVM (Jensen and Yaksh, 1986b; Hammond and Yaksh, 1984; Kuraishi, Harada, Satoh and Takagi, 1979; Barbaro, Hammond and Fields, 1985; Satoh, Akaike, Nakazawa and Takagi, 1980).

It has been generally accepted that serotonin plays a crucial role in the mediation of supraspinal opioid antinociception (Peroutka, 1988; Peroutka, Schmidt, Sleight and Harrington, 1990). In fact, the RVM is the major source of the dorsal horn 5-HT (Pazos and Palacios, 1985; Pazos, Probost and Palacios, 1987; Glennon, 1987; Gozlan, Mestikawy, Pichat, Glowinski and Hamon, 1983). Serotonin was initially implicated in the control of nociceptive thresholds and opiate analgesia following lesions placed in the medial forbrain bundle (Tilson and Rech, 1974; Vogt, 1974; Gorlitz and Frey, 1972). Use of 5-HT receptor blockers appeared to establish a role for 5-HT in descending modulation in pain inhibition, particularly for certain forms of supraspinal opiate and opioid analgesia (Mayer and Price, 1976; Yaksh and Rudy, 1978). Serotonin synthesis inhibition with para-chlorophenylalanine attenuated the antinociceptive effect of morphine (Tenen, 1968), which could be reversed with the 5-HT precursor, 5-hydroxytryptophan (5-HTP). Further, it has been shown that antinociceptive PAG stimulation results in the release of 5-HT from the spinal cord (Yaksh and Tyce, 1979). Petrouka (1988) and colleagues identified the existence of at least four subpopulations of the $5\text{-HT}_1$ receptor subtype (categorized as a-d) and the $5\text{-HT-}_2$ and $5\text{-HT}_3$ receptor subtypes. Evaluation of the role of spinal $5\text{-HT}_1$ receptors in nociception revealed that stimulation of these receptors

produce hyperalgesia rather than analgesia (Millan and Coelpaert, 1991a,b). Further, administration of either the general 5-HT antagonist, methysergide, the $5HT_2$ antagonist, ritanserin or the $5HT_3$ antagonist, ICS205930 into the RVM significantly attenuated morphine analgesia elicited from the PAG, implicating serotonergic $5HT_2$ and $5HT_3$ synapses in this pathway (Kiefel et al., 1991,1992). The above-mentioned studies using serotonergic antagonists further demonstrated the involvement of descending serotonergic pathways in the mediation of supraspinal opioid antinociception.

In addition to its direct action at spinal levels, norepinephrine influences nociceptive modulatory neurons in the RVM. Norepinephrine-containing fiber terminals are present throughout the RVM including both $alpha_1$ and $alpha_2$ binding sites. Further administration of $alpha_2$ noradrenergic receptor antagonists produces antinociception that is modulated by noradrenergic and serotonergic synapses in the spinal cord (Hammond et al., 1980 a,b; Sagen and Proudfit, 1985, Sagen, Winker and Proudfit, 1983; Henderson, Hughes and Kosterlitz, 1972).

Finally, neurons containing neurotensin and somatostatin may also constitute components of the descending endogenous pain control system (Beitz et al., 1983). Neurotensin-binding sites have been localized to the NRM and NRGC and other areas of the medulla, and therefore may be involved in the nociceptive influence of the PAG.

**F. Functional InterRelationship between Opioid systems in the Neuraxis.**

When discussing the relationship between two or more sites in the endogenous pain control system, one must examine either the drug-drug and/or site-site interactions. Thus, the primary question behind the present research concerned which supraspinal structures were interacting with each other, to what degree was this interaction, and which pharmacological agonists were involved. A variety of studies have demonstrated the sites of antinociceptive action of morphine, e.g. the PAG, the DLP and the RVM which includes the NRM and NRGC.

In 1980, Yeung and Rudy provided evidence for antinociceptive synergism between spinal (i.t.) and supraspinal (i.c.v.) morphine, and indicated that such a multiplicative interaction may play a role in the antinociception of systemic morphine. Yeung and Rudy (1980a) found that concurrent injections of morphine into the spinal cord and into the third cerebral ventricle of rats produced a multiplicative antinociceptive effect, which was maximal when equivalent doses of morphine were injected into both sites. These investigators proposed that the synergistic effect was responsible for the antinociception observed following systemic morphine administration. These authors (Yeung and Rudy, 1980b) also found further important results. First, naloxone injected into the spinal

subarachnoid space of the rat, which prevented diffusion of the antagonist to the supraspinal structures, blocked the antinociceptive effect of systemically-administered morphine. However, at higher morphine doses, naloxone failed to attenuate the antinociception. These and other authors (Roerig, Hoffman, Takemori, Wilcox and Fujimoto, 1991; Roerig and Fujimoto, 1989; Siuciak and Advokat, 1989) found these results to be intriguing since the usual assumption is that the effects of morphine at spinal and supraspinal sites are additive, and that the removal of one source of agonism will reveal the extent to which the other source contributes to antinociception.

Several authors (Yeung and Rudy, 1980a, Siuciak and Advokat, 1989; Fujimoto, Roerig and Tseng, 1988; Miyamoto et al., 1991; Jensen and Yaksh, 1986a) examined the opiate system by the use of i.c.v. and intrathecal (i.t.) methods, and have demonstrated that opiate synergy can be obtained from concurrent PAG and intrathecal injections. However, they do not rule out the possibility that a multiplicative effect could also be mediated by other intracerebral sites. It also remains to be seen whether morphine injection into supraspinal sites, which do not independently elicit antinociception, would also induce a synergistic effect with concurrent intrathecal injections. If the supraspinal action of intracerebral morphine injections could be dissociated from the synergistic interaction produced by

concurrent spinal administration, it might be possible to specify the origin of the descending pathways that mediate opiate synergy and determine whether they are the same as those which mediate antinociception produced by systemic morphine administration. Recent attempts (Roerig, O'Brien, Fujimoto and Wilcox, 1984; Roerig and Fujimoto, 1988; Roerig, Fujimoto and Tseng, 1988; Roerig and Fujimoto, 1987, 1989; Bodnar et al., 1991) have clarified some of the anatomical and pharmacological loci controlling descending pathways responsible for either tonic inhibition of spinal nociceptive reflexes, or antinociception produced by electrical brain stimulation or supraspinal morphine. Comparisons between descending pathways that support a multiplicative relationship and those responsible for other types of supraspinally-mediated inhibitions of spinal reflex pathways may be useful in resolving these issues.

Although the mechanism responsible for opiate synergy is unknown, recent results (Siuciak and Advokat, 1989) have suggested that the antinociceptive effect of intrathecal morphine in intact animals is tonically suppressed by descending inhibitory input. Also, these and other authors (Siuciak and Advokat, 1989; Fujimoto et al., 1988) propose that supraspinal morphine administration decreases this inhibitory input. The removal of descending inhibition allows the antinociceptive effect of spinal morphine to be expressed, resulting in increased potentcy of intrathecal morphine.

Although this interpretation of opiate synergy is similar to other authors (LeBars, Dickerson and Besson, 1983), it conflicts with the prevailing view that morphine acts in the brain in an opposite manner, that is to increase descending inhibition of spinal nociceptive processing. Additionally the proposal presented here postulates that it is the antinociceptive action of morphine at supraspinal sites that is under inhibitory control, rather than nociceptive input per se.

Miyamoto et al., (1991) showed that morphine antinociception, after various routes of administration, occurred on the tail-flick test, and that morphine content revealed that supraspinal and spinal morphine interacted multiplicatively to produce antinociception after i.c.v and i.t. and subcutaneous administration.

**G. Rationale.** In order to more accurately establish the role of opiate receptors in supraspinal analgesia, recent investigations have turned with increasing frequency to methods which permit the application of drugs directly to single neurons or specific neuronal pools. One difficulty with the i.c.v. method of administration was the wide distribution of drug throughout the ventricular system. A second disadvantage is the lack of knowlege for precise measurement of drug taken up by the area receptors.

An alternative method for bypassing the blood brain barrier and administering a substance into a localized

region of the central nervous system is the intracerebral microinjection technique (i.c.). By implanting a stainless-steel guide cannula into a selected locus within the CNS, an extremely precise volume of drug solution can be infused directly into the tissue. Therefore, a circumscribed neuronal pool was affected. If the injected solution diffused and gained wide access to other structures, the usefulness of the data would be compromised and the apparent locus of action of morphine would be misinterpreted (Yaksh, 1979; Yaksh and Rudy, 1977, 1978). Yaksh, Yeung and Rudy (1976) demonstrated several advantages of the i.c. method of administration, most importantly i.c. injections allow for possibility to recruit a sufficient number of neurons to produce a behavioral response. Also, since narcotics elicit antinociception by activation of quite specific pharmacological receptors, and since potent and highly specific antagonists of the receptor interaction were available, it was possible to ascertain whether the effect elicited was mediated by classical opiate receptors.

Pert and Yaksh (1974) examined morphine antinociception by various routes of administration (i.c.v, i.t., and i.c.) and found that morphine injected directly into the central gray was ten times more potent when given i.c. than administered into the third ventricle or cerebral aqueduct (i.c.v.), and that i.t. administration was less likely to activate a precise spinal region, therefore working on

regions not only activated by opiates. Jacquet (1973) revealed i.c. injections of morphine differ significantly from i.c.v injections. It was shown that i.c. injections of morphine have effects significantly different from those of systemic injections, similar to the results of centrally acting drugs. In addition, several authors (Criswell, 1976; Nishimura, Recht and Pasternak, 1984) have indicated that i.c. microinjection, when used with care, is a powerful method of investigating morphine action by assessing a specific set of neurons rather than a large neuronal pool. Unlike i.c.v. and i.t. injections, i.c. minimized diffusion to other sites, and allowed for precise injection of dose to the site of interest.

Indeed, one would expect that systemically-administered morphine would produce an approximately equal degree of agonism at spinal and supraspinal sites of action. It was of considerable interest to note that data revealed concurrent i.t. and i.c.v. injections of morphine interacted in a supra-additive manner in both of the above reports, and that the strength of their mutual potentiation was apparently at the 1:1 ratio of supraspinal to spinal dosage.

Still, the question of which sites are interacting with each other exists. Yeung and Rudy (1980a) proposed an efferent-interaction model which assumed that spinal and supraspinal substrates each contribute a fixed proportion of the antinociceptive effect produced by systemically

administered morphine. In other words, each substrate should yield an independent antinociceptive effect and, when combined with the complementary substrate, it should yield the total observed antinociceptive response. Therefore, the efferent-interaction model of opiate action was not completely correct, especially since one deduced from the dose-response data and isobolographic analysis that supra-additivity of effects existed. Furthermore, antinociception produced by joint application of spinal and supraspinal morphine was greater than the sum of the effects produced by the same doses administered individually (Yeung and Rudy, 1980a).

In an earlier study, data revealed that low to moderate doses of systematically-administered morphine were less capable of mediating antinociception by independent actions and that the antinociception depended upon a multiplicative interaction between narcotic agonisms expressed at spinal and supraspinal sites of action. Yet, it remained a possibility that neither the spinal nor the supraspinal narcotic-sensitive substrate was capable of mediating antinociception in the total absence of narcotic agonism at the other substrate. Indeed, this implied that the interaction between spinal and supraspinal narcotic agonisms associated with systemically-injected morphine was multiplicative at all doses rather than at low to moderate doses only.

Thus, a major reason for undertaking the present dissertation was to ascertain the relative importance of the supraspinal sites of narcotic action in mediating the antinociceptive effects of i.c. administered morphine. Investigations relevant to this issue revealed that neither locus of action (spinal nor supraspinal) was considered predominant, and it remained evident that further information was needed concerning pairs of supraspinal sites e.g., PAG, DLP, and RVM.

Given that antinociceptive synergy occurred between supraspinal and spinal opiate-sensitive sites, and given that anatomical and physiological connections existed between these supraspinal sites, it was the central premise of this dissertation that antinociceptive synergy should occur following morphine microinjections into pairs of the following sites: PAG/RVM, DLP/RVM and PAG/DLP. To this end, the following experiments were performed. In Experiment 1, detailed dose-response curves for morphine antinociception were performed for the PAG, RVM and DLP. This was necessary to allow the choice of appropriate doses for ascertaining whether or not synergy had occurred. Then, simultaneous morphine microinjections into selected pairs of the PAG, RVM and DLP were evaluated, including the construction of dose-response curves for each site in the simultaneous condition. Given that there is sensitivity to the indiscriminate use of animals in research, one would try

to do a within subjects design, in that more than one pain test would be tested on a given animal across a given time course. However, morphine and enkephalin peptide analogues produce different dose response curves as a function of the nociceptive test, and since the study of synergy depends upon the precise calculation of sub-analgesic doses, this became problematic for within dose-testing. Therefore the present study only used one specific test sensitive to opiates, the tail-flick test.

Following the determination of synergistic interactions, pretreatment with the the highly selective $mu_1$ antagonist, naloxonazine was administered to determine the opiate receptor responsible for the synergy. Given that PAG projections to the spinal cord are sparse and are not simply monoaminergic, this aspect of study emphasizes the probability that functionally excitatory projections from the PAG to cells of origin in the pons and medulla are an important intermediary link.

In Experiment 2, additional opioid agonists were tested in order to further explain the pharmacological basis of the supraspinal pain inhibitory system. As mentioned, enkephalinergic-immunoreactive neurons project from the PAG to the NRM, (Beitz, 1982a), and also serve as intrinsic interneurons in the NRM (Lewis et al., 1985). Antinociceptive responses are elicited from the NRM following morphine, mu-selective agonists and delta-

selective agonists (Azami et al., 1982; Bodnar et al., 1988; Satoh, Kubota, Iwama, Wada, Yasui, Fujibayashi and Takagi, 1983; Zorman et al., 1981) which are consistent with the observations that both mu and delta receptors are present in these structures (Goodman et al., 1980; Lewis, Cannon and Liebeskind, 1980; Moskowitz and Goodman, 1985a,b). Therefore, this and the subsequent experiment will further explain the role of specific opioid agonists in the mesencephalic/mylencephalic areas of the brain. In the second experiment, the mu receptor agonist, DAMGO, the $kappa_1$ receptor agonist, U50488H and the $delta_1$ receptor agonist, DPDPE were respectively examined in the PAG and RVM for antinociception. These agonists were then evaluated at specific doses in the selected pairs of sites to determine whether they produced multiplicative interactions. Experiment 3 determined whether the $delta_2$ agonist, deltorphin displayed antinociception in the PAG and RVM, alone and in conjunction with the above mentioned agonists.

## General Methods

**Subjects.** Male albino Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, 80-120 days of age) weighing between 320-750 grams, were housed individually in plastic cages in the Queens College Vivarium Facility and provided with Purina Rat Chow and water ad libitum. All animals were maintained on a 12 hour light : 12 hour dark cycle at ambient room temperatures between 21° and 25°C.

**Surgery.** Rats were anesthetized with chlorpromazine HCl (3 mg/kg, IP) 20 minutes prior to ketamine HCl (100 mg/kg, IM) and stereotaxically implanted (Kopf Instruments) with two stainless steel guide cannulae (28 gauge, Plastic Products Co., Roanoke, VA) aimed 0.1 mm dorsal to the sites of interest. The stereotaxic coordinates were taken from Paxinos and Watson (1986). With the incisor bar always set at -5 mm, the coordinates for the periaqueductal gray were 0.3-0.6 mm anterior to the lambda suture, 1.5-2.0 mm lateral to the sagittal suture, 6.8-7.0 mm from the top of the skull, and angled 12° toward the sagittal plane. Coordinates for the dorsal lateral pons were 1.5 mm posterior to the lambda suture, 3.0 mm lateral to the sagittal suture, 7 mm from the top of the skull, and angled toward the sagittal plane at 15.5°. Coordinates for the RVM were 10.8-11.3 mm posterior to the bregma suture, 0.0-0.7 lateral to the midline, and 9.5-11 mm from the top of the skull. The NRGC coordinates were as follows: 10.8-11.3 mm

posterior to the bregma suture, 0.0-0.7 mm lateral to the midline, and 9.5-11 mm from the top of the skull. Cannulae were secured to the skull with three anchor screws and dental acrylic. All guide cannulae were kept patent with a dummy cannulae (Plastic Products). All animals were allowed one week to recover from cannulae surgery and clearing of the anesthetic before the initiation of behavioral testing.

**Pharmacological Conditions.** The first opioid agonist in this study was morphine HCl which was infused intracerebrally into the PAG, DLP, RVM or pairs of these sites (Experiment 1). Morphine (Pennick Laboratories) was dissolved in normal 0.9% saline. DAMGO (Research Biochemicals), U50,488H (Upjohn), DPDPE (Research Biochemicals) were dissolved in normal 0.9% saline. Deltorphin II (Penninsula Laboratories) was dissolved in distilled water with 1 $\mu$l of glacial acetic acid to prevent drug coagulation. All opioid agonist infusions were in 1 $\mu$l volumes injected continuously through a Hamilton syringe, which was connected to an internal cannula (33 gauge, Plastic Products) by polyethylene tubing. All intracerebral microinjections proceeded over a 30 second time interval. Opioid injections were separated by a one-week intervals to minimize potential tolerance effects. Multiple intracerebral injections of opioid agonists into the same site can maintain a comparable analgesic effect across time (Yaksh et al., 1976; Bodnar et al., 1988, 1991). To

complete the pharmacology of Experiment 1, the $\mu_1$ receptor antagonist, naloxonazine was used. Naloxonazine (10 mg/kg, synthesized by Dr. G.W. Pasternak) was dissolved in normal 0.9% saline and was infused intravenously 24 hours prior to testing. Alternatively, a number of studies have used smaller concentration volumes. Yaksh and collegues (1986) used comparable 1 $\mu$l amounts in order to aquire complete drug solubility. Although, the 1 $\mu$l volume slightly increases the possibility of the spread and diffusion over a larger area, it is primarily a cost-benefit relationship for using the 1 $\mu$l volume.

**Nociceptive Apparatus.** All animals were tested on the tail-flick test (D'Amour and Smith, 1941). The tail-flick test was used to test spinal reflexes, and correlates well in measuring efficacy of analgesics and clinical pain. Further, this test was primarily employed in assessing the synergistic interactions between ventricular and spinal sites, and in the physiological substrates of RVM neurons. This test measured reactivity to thermal heat directed at the tail by the use of a stimulus source (IITC Company) which was positioned 8 cm dorsal and 3-9 cm proximal to the tip of a lightly restrained animal. The onset of the radiant heat stimulus activated a digital timer which was stopped by the withdrawal of the animal's tail. The tail-flick latency was described as the time which elapsed between the onset of the radiant heat stimulus and the

withdrawal response of the animals. The intensity of thermal stimulus was set to produce stable baseline tail-flick latencies between 1.8 and 3.0 seconds. In order to avoid tissue damage, a trial was automatically terminated if a response did not occur within 12 seconds. All animals displayed consistent latencies in baseline and vehicle testing, and did not appear subject to desensitization.

**Statistical Analysis.** All data were subjected to analyses of variance to determine whether opioid agonists at particular doses administered into either one or two sites produced an antinociceptive effect relative to baseline. Analyses of variance were performed on actual latencies. Dunnett comparisons were used to determine individual effects.

Analyses of peak and total opioid agonist dose response curves were performed in the first experiment using linear regression analyses which determined the slope, intercept and the $ED_{50}$ of opioid antinociception from each site individually and from multiple sites following simultaneous administration. Peak antinociceptive responses occurred 30 min following morphine administration and 15 min following selective opioid agonist administration. These time points, therefore were used to assess peak effects. Total antinociceptive effects were ascertained by summing the magnitudes of antinociception across the total time of testing. For regression analyses, all scores were converted

to Maximal Percentage Effect (MPE) which was derived by subtracting the baseline scores from each experimental score and dividing this by the difference between the baseline score from the 12 sec cut-off score. The resultant value was then multiplied by 100. The MPE has been repeatedly used in both synergy (e.g., Yeung and Rudy, 1980a; Roerig et al., 1987; Miyamoto et al., 1991), pharmacology (e.g., Bodnar et al., 1988, 1991) and physiology (e.g. Fields et al., 1991) studies. The regression analyses then determined whether the dose-response curves were significantly different from each other.

**Histological Procedures.** After experimental testing, anesthetized (Euthanasia, H. Schein) rats received a transcardiac perfusion with 0.9% normal saline followed by 10% buffered formalin. Coronal (40-$\mu$m) sections, stained with cresyl violet, and cannula placements were examined by light microscopy by an observer uninformed with respect to the behavioral data. Only animals with confirmed cannulae placements were included in the data analysis.

**EXPERIMENT 1: Synergistic Brainstem Interactions for Morphine Antinociception.**

The proposed neural substrates of supraspinal opioid antinociception originate in the midbrain PAG, synapse in the RVM, and project to the substantia gelatinosa of the spinal cord through the dorsolateral funiculus (Fields and Basbaum, 1978; Basbaum and Fields, 1984). The NRGC and the NRGC, pars alpha (Aimone, Bauer and Gebhart, 1988; Azami et al., 1982; Sandkuhler and Gebhart, 1984a; Satoh et al., 1983) and the DLP (Bodnar et al., 1988, 1991) have subsequently been implicated in the medullary mediation of supraspinal opioid antinociception. It is not clear whether supraspinal antinociception elicited from each of these sites is the result of punctate activation of each site alone or is the result of activation of an interconnected system. Thus, these studies examined whether the antinociception elicited by microinjections of morphine into the PAG, DLP or RVM alone can interact when sub-antinociceptive doses of morphine are microinjected into pairs of sites. Thus, regional antinociceptive interactions between the PAG and RVM, the RVM and DLP and the PAG and the DLP were examined. In addition, full dose-response curves for such interactions were performed such that a fixed sub-antinociceptive dose was placed in one site, and a range of sub-antinociceptive doses was placed in the second site. Then this procedure was reversed. Finally, this experiment

examined whether intrinsic brainstem $mu_1$ receptor systems modulated the synergistic interactions through the use of naloxonazine antagonism.

## METHODS

Single-Site Protocol: Rats were implanted with two cannulae aimed at either the PAG, the RVM and/or the DLP. Each rat received a maximum of four single microinjection conditions at weekly intervals. Dose-response curves were assessed for morphine antinociception at each site (Table 1). All microinfusions were administered in 1 $\mu$l volumes, and vehicle and morphine microinjections were in 0.9% normal saline. Tail-flick latencies were determined at 30, 60, 90 and 120 min following each microinjection. This time course of testing of morphine was chosen since it is commonly used in previous studies (Kiefel et al., 1991, 1992). In several analyses the term "peak effect" was used and occurred at the 30 minute interval. This was operationally defined and is conceivable that the largest changes in latencies may have occurred shortly before or after this tiem interval.

Double-Site Protocol: Rats were implanted with two cannulae aimed at either the PAG, the RVM and/or the DLP. Each rat received a maximum of four double microinjection conditions at weekly intervals. The single-site protocol indicated that a 1 $\mu$g dose of morphine was ineffective in producing significant antinociception from PAG, RVM and DLP placements alone. Thus, in the double-injection protocol, a

Table 1. Summary of morphine microinjections into either the PAG alone, the RVM alone or the DLP alone.

| Condition | Sample Size |
|---|---|
| A. PAG Placements. | |
| Vehicle | 10 |
| 1 μg | 6 |
| 1.5 μg | 6 |
| 2 μg | 6 |
| 3μg | 5 |
| 5μg | 4 |
| B. RVM Placements. | |
| Vehicle | 23 |
| 1μg | 5 |
| 1.5μg | 3 |
| 2μg | 12 |
| 3μg | 11 |
| 5μg | 4 |
| 10μg | 4 |
| C. DLP Placements. | |
| Vehicle | 17 |
| 1μg | 6 |
| 1.5μg | 6 |
| 3μg | 7 |
| 5μg | 8 |
| 10μg | 4 |

PAG: Periaqueductal Gray; RVM: Rostro-ventral medulla; DLP: Dorsolateral Pons

1 μg dose of morphine was administered into the first site of a pair, and a 1 μg dose of morphine administered into the second site of a pair simultaneously. This generated two-way dose-response curves. In some animals, the 1 μg dose of morphine was held constant in the first site, and the dose of morphine administered into the second site was varied. In other animals, the 1 μg dose of morphine was held constant in the second site, and the dose of morphine administered into the first site was varied. Table 2 summarizes the double-site injection paradigm for PAG-RVM placements, for DLP-RVM placements and for DLP-PAG placements. Tail-flick latencies were assessed 30, 60, 90 and 120 min after the double-site injections.

To evaluate whether potential synergy between PAG and RVM placements involved the $mu_1$ receptor, five additional rats were anesthetized and implanted with jugular catheters as well as PAG and RVM cannulae. Rats received simultaneous intracerebral injections of 1 μg doses of morphine into the PAG and RVM to determine the presence of synergy. One week thereafter, the $mu_1$ antagonist, naloxonazine (10 mg/kg: Hahn et al., 1982; Bodnar et al., 1988, 1991), was administered 24 h prior to simultaneous intracerebral injections of morphine (1 μg) into the PAG and RVM. Tail-flick latencies were assessed 30, 60, 90 and 120 min after the intracerebral injections.

TABLE 2. Summary of double-site morphine microinjections in the PAG and RVM, the DLP and RVM and the DLP and PAG.

A. PAG-RVM Combination.

| PAG Condition | RVM Condition | Sample Size |
|---|---|---|
| Vehicle | Vehicle | 19 |
| Morphine 1μg | Morphine .2μg | 6 |
| Morphine 1μg | Morphine .5μg | 5 |
| Morphine .3μg | Morphine 1μg | 4 |
| Morphine .7μg | Morphine 1μg | 6 |
| Morphine 1μg | Morphine 1μg | 7 |

B. DLP-RVM Combination.

| DLP Condition | RVM Condition | Sample Size |
|---|---|---|
| Vehicle | Vehicle | 10 |
| Morphine 1μg | Morphine .2μg | 6 |
| Morphine 1μg | Morphine .5μg | 6 |
| Morphine .3μg | Morphine 1μg | 4 |
| Morphine .7μg | Morphine 1μg | 5 |
| Morphine 1μg | Morphine 1μg | 7 |

C. DLP-PAG Combination.

| DLP Condition | PAG Condition | Sample Size |
|---|---|---|
| Vehicle | Vehicle | 9 |
| Morphine 1μg | Morphine .2μg | 2 |
| Morphine 1μg | Morphine .5μg | 4 |
| Morphine .2μg | Morphine 1μg | 6 |
| Morphine .5μg | Morphine 1μg | 7 |
| Morphine 1μg | Morphine 1μg | 7 |

## Results

**Histological Verification:** Mesencephalic cannula placements in the PAG group (Figure 1) were all localized in the lateral, ventral and ventro-lateral quadrants of the PAG and immediately-adjacent dorsal raphe nucleus. These placements were as far rostral as the III cranial nerve nucleus and as far caudal as the dorsal raphe nucleus. Cannula placements in the DLP group (Figure 2) were localized in several structures. The most rostral placements in this group were in the central tegmental tract which includes the dorsal noradrenergic bundle. Whereas a second group of placements were in the locus coeruleus, a third group of placements were in and around the parabrachial nuclei. Finally, cannula placements in the RVM group (Figure 3) were localized in either the NRM, the NRGC or the NRGC, pars alpha as far rostral as the genu of the VII cranial nerve and as far caudal as the nucleus of the VII cranial nerve. The cannula placements in each of the sites were quite punctate, and produced minimal tissue damage similar to that observed in photomicrographs in a previous study (Kiefel et al., 1992).

**Single-Site Injections:** For PAG placements, significant differences in tail-flick latencies were observed among doses ($F(5,31)= 37.89$, $p<.0001$), across times ($F(3,93)= 163.05$, $p<.0001$) and for the interaction between doses and times ($F(15,93)= 27.70$, $p<.0001$). Morphine significantly

Figure 1. Histological verification of cannula placements using the atlas of Paxinos and Watson (1986). The figure depicts mesencephalic cannula placements (Bregma: -6.8 mm to -8.0 mm) in which PAG-morphine (2-5 μg) elicited an analgesic response on the tail-flick test. Closed circles depict mesencephalic placements of rats within the PAG. Mesencephalic cannula placements in the PAG group were all localized in the lateral, ventral and ventro-lateral quadrants of the PAG and immediately-adjacent dorsal raphe nucleus. These placements were as far rostral as the III cranial nerve nucleus and as far caudal as the dorsal raphe nucleus. The numbers adjacent to the closed circles indicate the number of rats with cannula placements in that site, and the three numbers below the figure represent the corresponding plate number in the atlas of Paxinos and Watson.

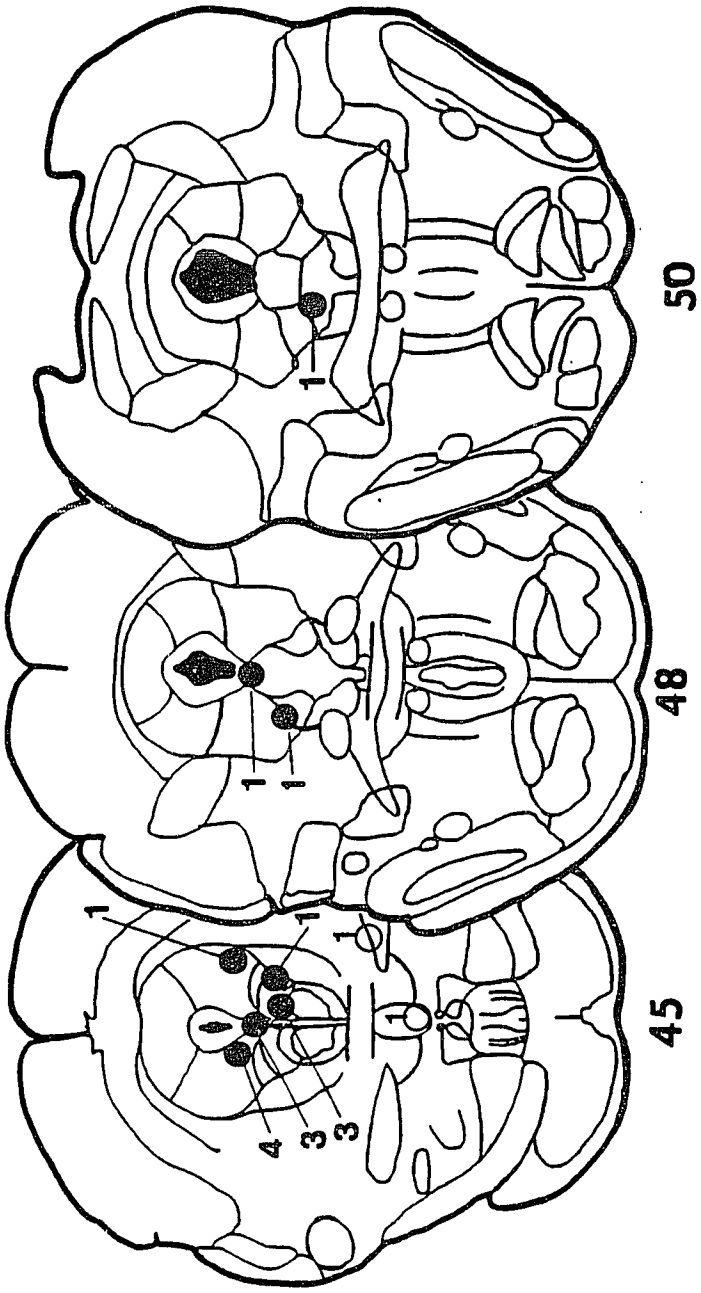

50
48
45
1
1
1
1
1
1
4
3
3

Figure 2. Histological verification of cannula placements using the atlas of Paxinos and Watson (1986). The figure depicts pontine cannula placements (Bregma: -8.0 mm to -9.3 mm) in which DLP-morphine (3-10 μg) elicited an analgesic response on the tail-flick test. Closed circles depict cannula placements in the DLP which were localized in several structures. The most rostral placements in this group were in the central tegmental tract, which included the dorsal noradrenergic bundle. A second group of placements were in the locus coeruleus, and a third group of placements were in and around the parabrachial nuclei. The numbers adjacent to the closed circles indicate the number of rats with cannula placements in that site, and the four numbers below the figure represent the corresponding plate number in the atlas of Paxinos and Watson.

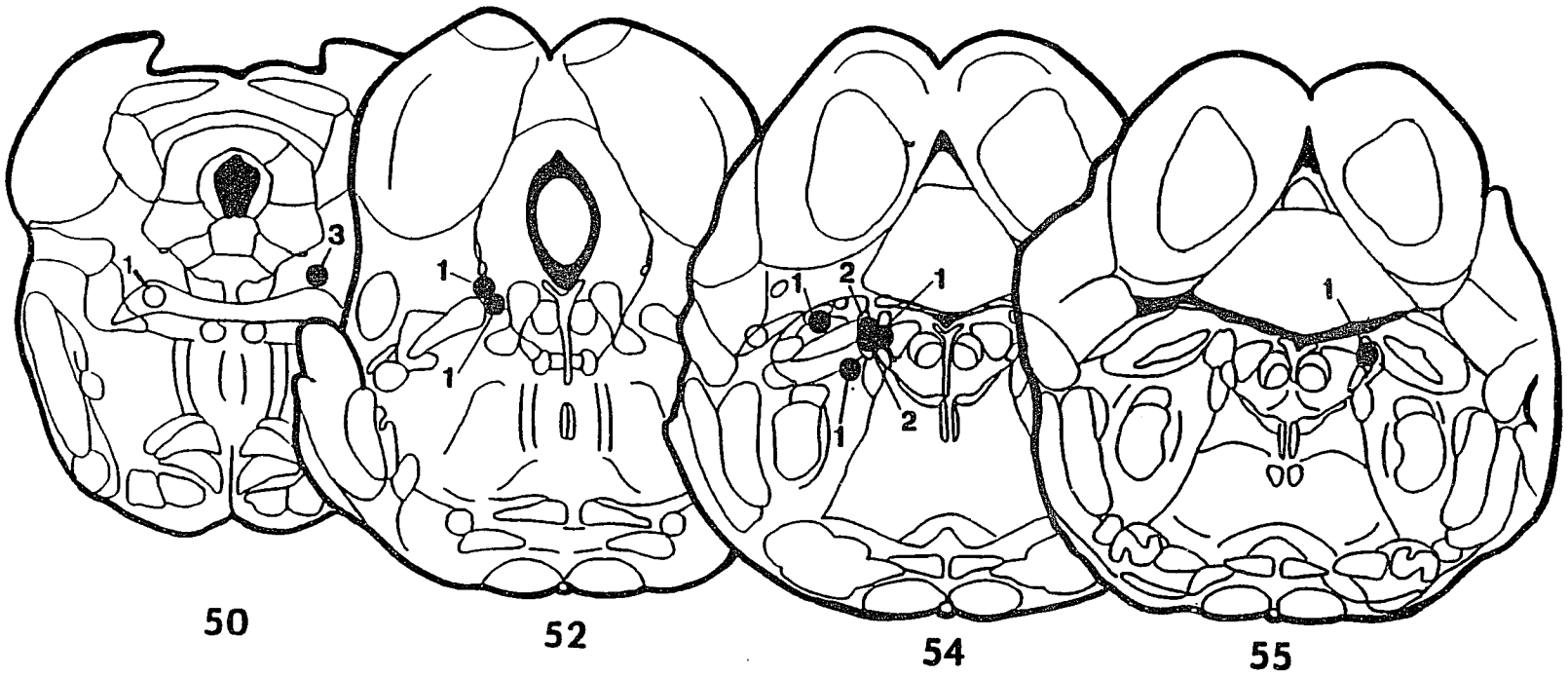
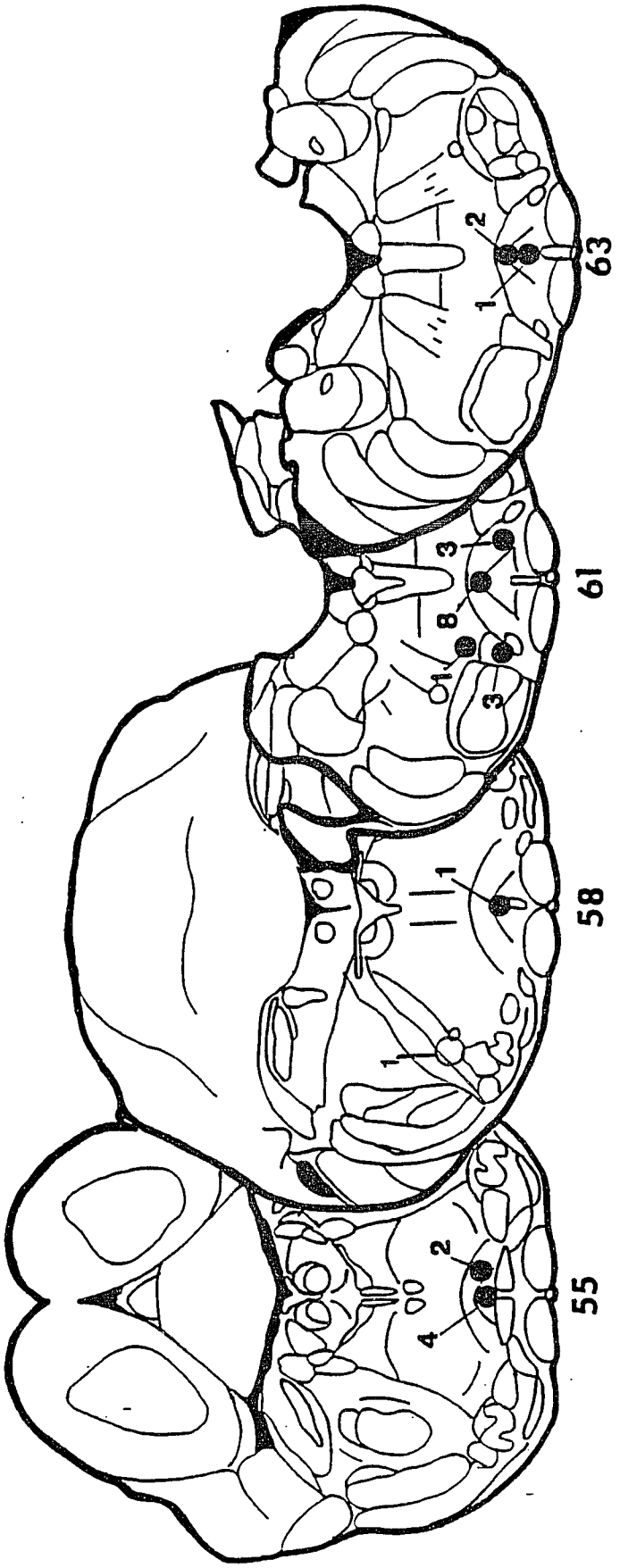

1
3
1
1
1
2
1
1
2
1
1
50
52
54
55

Figure 3. Histological verification of cannula placements using the atlas of Paxinos and Watson (1986). The figure depicts medullary cannula placements (Bregma: -9.3 mm to -11.3 mm) in which NRM-morphine (2-10 $\mu$g) elicited an analgesic response on the tail-flick test. The closed circles depict rats with medullary cannulae localized either in the NRM, NRGC or NRGC, pars alpha. These placements were as far rostral as the the genu of the VII cranial nerve and as far caudal as the nucleus of the VII cranial nerve. The numbers adjacent to the closed circles indicate the number of rats with cannula placements in that site, and the four numbers below the figure represent the corresponding plate number in the atlas of Paxinos and Watson.


63
61
58
55
2
1
3
8
1
3
1
1
2
4

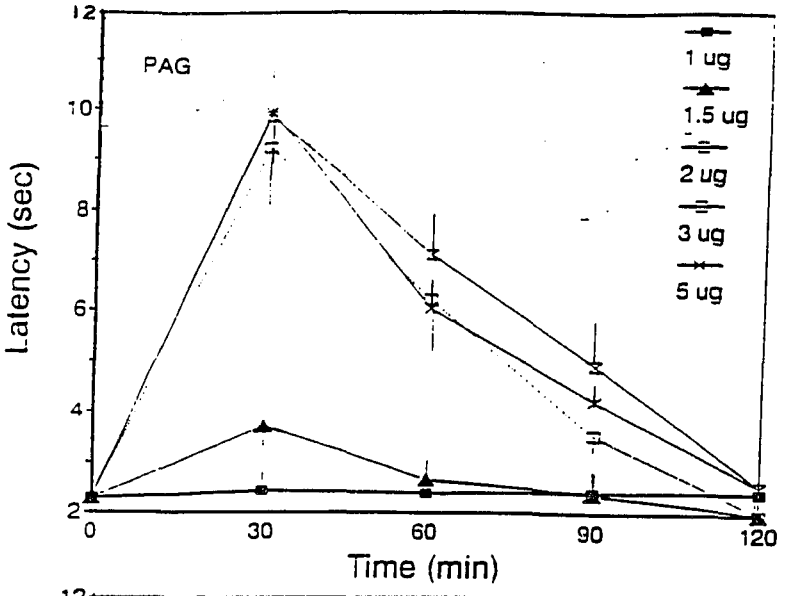
elevated latencies in a dose-dependent manner with a peak effects at 30 min and a duration of action of approximately 90 min (Figure 4a). Although the response was quite robust, the dose-response curve reveals a ceiling effect with latencies of approximately 10 sec at doses above 2$\mu$g (Figure 5a). The magnitude of mesencephalic morphine antinociception was similar in rats with rostral and caudal PAG placements, an effect consistent with previous studies (Kiefel et al., 1991,1992; Robertson and Bodnar, 1993).

For DLP placements, significant differences in tail-flick latencies were observed among doses ($F(5,42)= 22.74$, $p<.0001$), across times ($F(3,126)= 77.18$, $p<.0001$) and for the interaction between doses and times ($F(15,126)= 20.10$, $p<.0001$). Morphine significantly and dose-dependently increased latencies, with apeak effect at 30 minutes (Figures 4b and 5b). Inspection of the dose-reponse curves suggested that the potency of morphine in the PAG is approximately 4-fold greater than in the DLP. However, the responses in the DLP group varied as a function of cannulae site. Placements in the locus coeruleus or dorsal noradrenergic bundle displayed maximal antinociception (>12 sec) with morphine (5 $\mu$g) in three of six rats. Rats with parabrachial nucleus cannulae typically displayed a doubling of baseline latencies following morphine (5-7 sec).

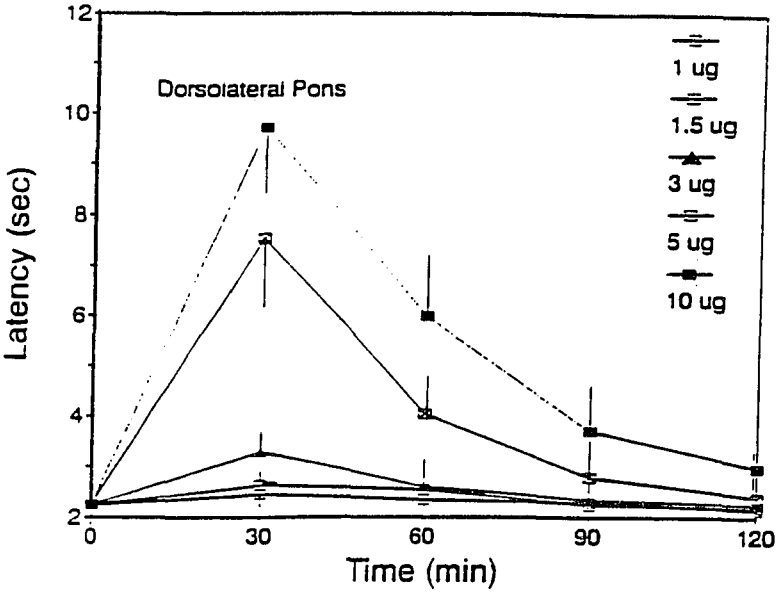
For RVM placements, significant differences in tail-flick latencies were observed across doses ($F(6,55)= 59.33$,

Figure 4. Time Action Curves of Single-Site Morphine Injections. Rats cannulated in the a) PAG, b) DLP, or c) RVM received the stated morphine injections. Results indicate mean-tail-flick latencies with S.E.M. bars. Significant increases in tail-flick latencies were observed across morphine doses, across time courses, and for the interactions between doses and times.

A


PAG
1 ug
1.5 ug
2 ug
3 ug
5 ug
Latency (sec)
Time (min)
0
30
60
90
120
2
4
6
8
10
12

B


Dorsolateral Pons
1 ug
1.5 ug
3 ug
5 ug
10 ug
Latency (sec)
Time (min)
0
30
60
90
120
2
4
6
8
10
12

C

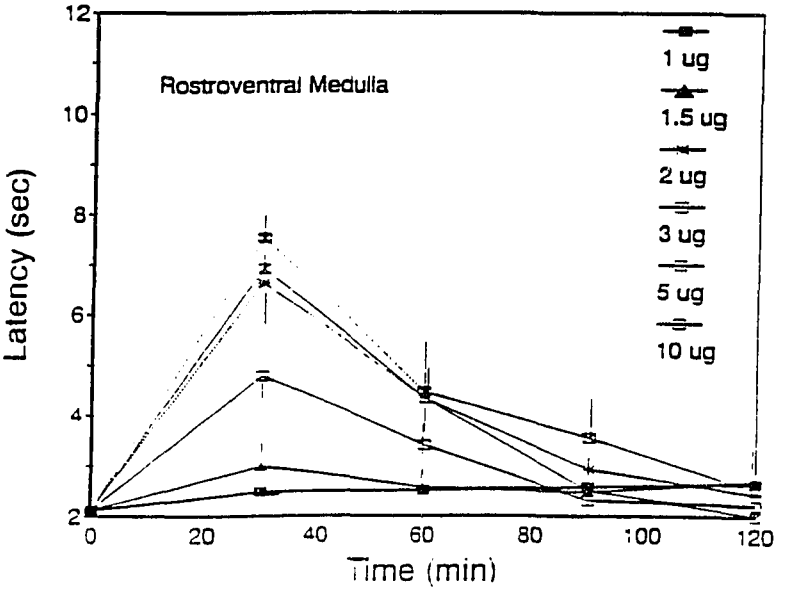
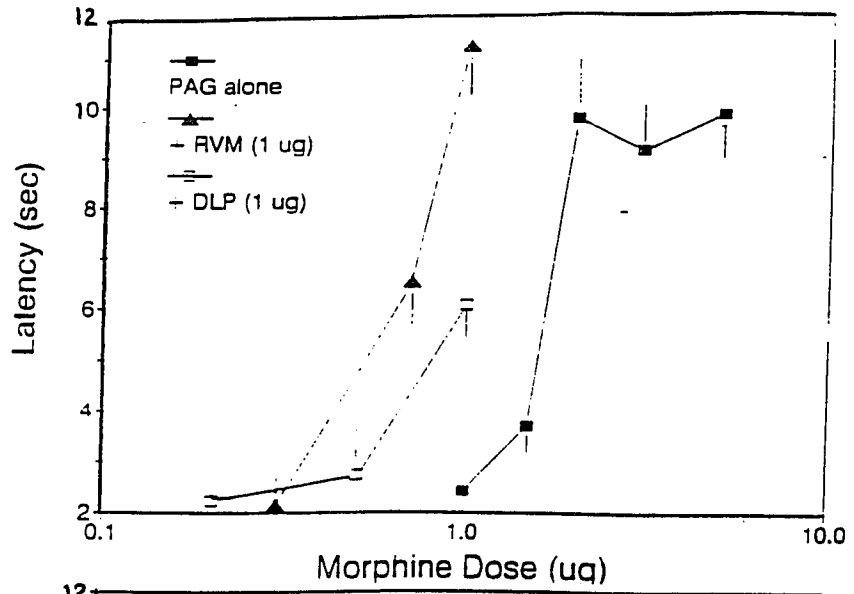
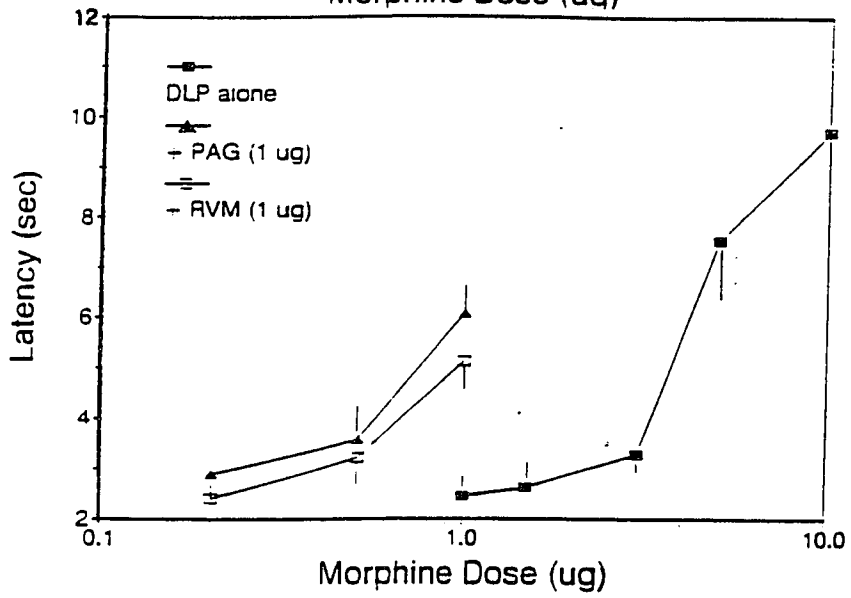

Rostroventral Medulla
1 ug
1.5 ug
2 ug
3 ug
5 ug
10 ug
Latency (sec)
Time (min)
0
20
40
60
80
100
120
2
4
6
8
10
12

Figure 5. Morphine Dose-Response Curves in Individual Sites and in Combinations. Cannulated rats received the specified dose of morphine in the stated region alone or in combination with a fixed 1 μg dose of morphine in the specified second region. Results indicate the mean peak tail-flick latencies with S.E.M bars. Figure 5a depicts the addition of morphine (1 μg) in the RVM which produced significant increases across PAG doses, across the time course, and significant increases for the interactions between doses and times. This fixed RVM dose significantly increased latencies when paired with the 0.7 and 1 μg PAG dose, but not the 0.3 μg dose. Similarly, the addition of (1 μg) in the DLP significantly increased latencies compared to 1 μg in the PAG alone. Figure 5b depicts the addition of morphine (1 μg) in the PAG and RVM which produced significant increases across DLP doses, across the time course and for the interactions between doses and times. The addition of morphine (1 μg) into either the PAG or RVM significantly elevated latencies when paired with a 1 μg dose in the DLP as compared to the DLP dose alone. Figure 5c depicts the addition of morphine (1 μg) in the PAG which produced significant increases across RVM doses, across the time course and for the interactions between doses and times. Coadministration of morphine in the DLP elicited significant antinociception from RVM placements following 0.5 and 1 μg doses.

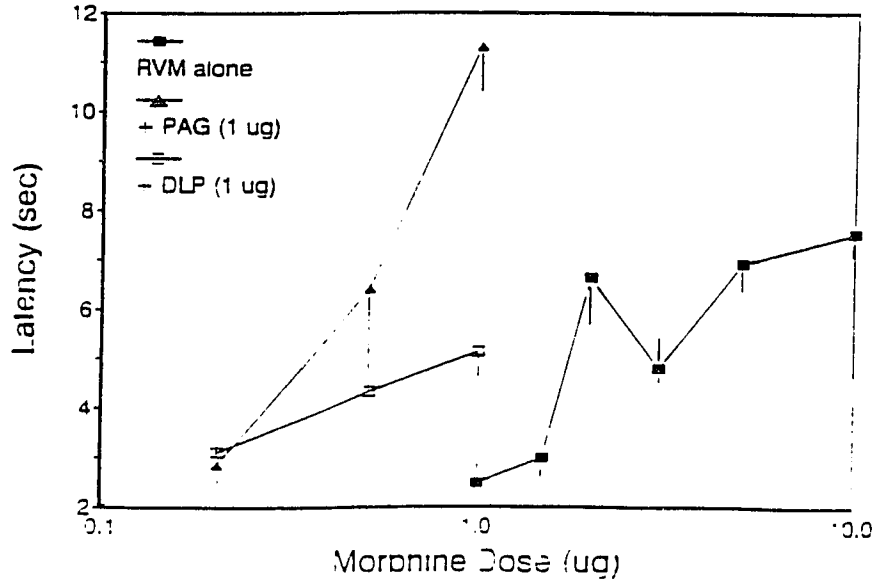
A


PAG alone
+ RVM (1 ug)
+ DLP (1 ug)
Latency (sec)
Morphine Dose (ug)
0.1
1.0
10.0
2
4
6
8
10
12

B


DLP alone
+ PAG (1 ug)
+ RVM (1 ug)
Latency (sec)
Morphine Dose (ug)
0.1
1.0
10.0
2
4
6
8
10
12

C


RVM alone
+ PAG (1 ug)
+ DLP (1 ug)
Latency (sec)
Morphine Dose (ug)
2
4
6
8
10
12

$p<.0001$), across times ($F(3,165)= 217.37$, $p<.0001$) and for the interaction between doses and times ($F(18,165)= 40.64$, $p<.0001$). Morphine was less efficaceous in the RVM, with a maximal latency between 7 and 8 sec (Figures 4c and 5c). The peak effect was seen at 30 min with a duration of approximately 60 min. These results are similar for all sites within the region and agree with prior studies (Bodnar et al., 1988). Although dose-response curves indicated a similar dose for half-maximal responses in both the RVM and the DLP, the RVM demonstrates a ceiling effect, implying a lower efficacy.

Both the time-action and the dose-response curves indicated that the latencies observed with a 1 $\mu$g dose of morphine were indistinguishable from baseline values in the PAG, DLP and RVM. The ineffectiveness of this low morphine dose was due to its lack of potency and not due to the location of the cannulae placements.

**Regional Interactions for Morphine**

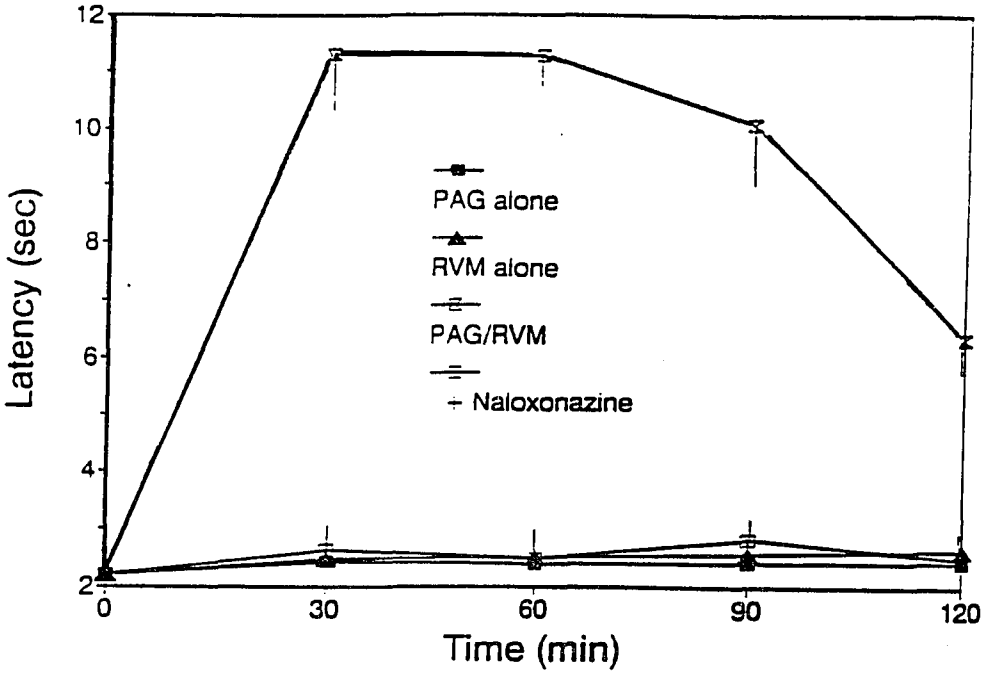
Coadministration of different morphine doses (0.2-1 $\mu$g) in the RVM paired with a 1 $\mu$g dose of morphine in the PAG significantly increased latencies relative to vehicle treatment across doses ($F(3,33)= 326.51$, $p<.0001$), across times ($F(3,99)= 46.86$, $p<.0001$) and for the interaction between doses and times ($F(9,99)= 20.41$, $p<.0001$). Coadministration of morphine (1 $\mu$g) into the PAG and the RVM produced a profound analgesic response, with a peak effect

close to the maximal observable response and a duration greater than 120 min (Figure 6a). This response, defined as the area under the time-action curve, far exceeded that seen with doses of morphine as high as 5μg in the PAG alone (Figure 4a) or 10μg in the RVM alone (Figure 4c).

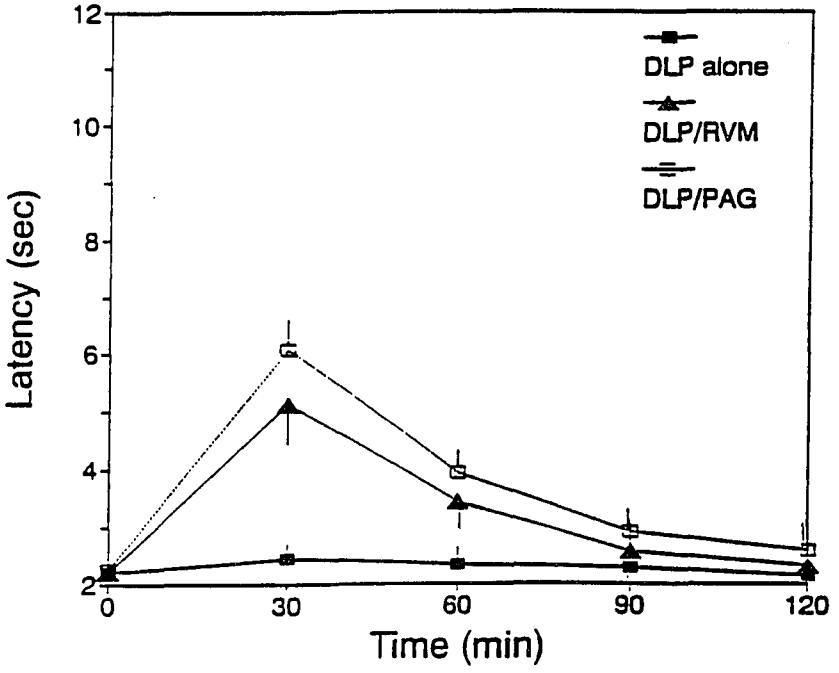
This study then examined the effects of a fixed 1μg dose in the RVM on the dose-response curve for morphine in the PAG. Coadministration of different morphine doses (0.3-1 μg) in the PAG paired with a 1 μg dose of morphine in the RVM significantly increased latencies relative to vehicle treatment across doses ($F(3,29)= 163.43$, $p<.0001$), across times ($F(3,87)= 26.75$, $p<.0001$) and for the interaction between doses and times ($F(9,87)= 12.01$, $p<.0001$). Administration of morphine in the RVM significantly shifted ($F(2,39)= 25.83$, $p<.001$) the PAG morphine dose-reponse curve to the left by over 3-fold (Figure 5a), strongly suggesting the presence of synergy. Similar results were observed when examining a fixed 1 μg PAG dose on the dose-response curve in the RVM (Figure 5b). In addition to significantly shifting the RVM dose-reponse curve approximately 10-fold to the left ($F(2,53)= 69.81$, $p<.0001$), the combination displayed no ceiling effect, which showed a maximal response over 11 seconds. The ceiling effect, an arbitrary effect, correlates with the cut-off latency which prevents tissue damage in the animal's tail. Thus, the low dose of morphine in the PAG also markedly increased the efficacy of morphine

Figure 6. Time-Action Curves of Fixed Morphine Doses in Single and Multiple Regions. Figure 6a. depicts low morphine dose (1 μg) injected into either the PAG, RVM or both regions, as indicated. Another group was treated with naloxonazine (10 mg/kg, i.v.) 24 hr prior to the testing with morphine into both regions. Results shown here are the means and S.E.M of tail-flick latencies and the combinations of morphine were significantly different from single regions or combined injections in naloxonazine-treated animals from 30-120 min. Figure 6b. depicts low morphine doses (1 μg) injected into the DLP alone or in combination with either RVM or PAG, as indicated. Results shown here are the means and S.E.M of tail-flick latencies and the combinations are significantly different from the DLP alone at the 30 min point.

A


12
10
8
6
4
2
Latency (sec)
PAG alone
RVM alone
PAG/RVM
+ Naloxonazine
0
30
60
90
120
Time (min)

B


12
10
8
6
4
2
Latency (sec)
DLP alone
DLP/RVM
DLP/PAG
0
30
60
90
120
Time (min)

in the RVM, which had a ceiling effect observed when examining a fixed 1 μg PAG dose on the dose-response curve in the RVM (Figure 5b). In addition to significantly shifting the RVM dose-reponse curve approximately 10-fold to the left ($F(2,53)= 69.81$, $p<.0001$), the combination displayed no ceiling effect, which showed a maximal response over 11 seconds. The ceiling effect, an arbitrary effect, correlates with the cut-off latency which prevents tissue damage in the animal's tail. Thus, the low dose of morphine in the PAG also markedly increased the efficacy of morphine in the RVM, which had a ceiling effect of only 7-8 seconds when morphine was injected there alone.

The other combinations also elicited a greater than additive response, but not nearly as robust as that observed between the PAG and the RVM. Coadministration of different morphine doses (0.2-1 μg) in the DLP paired with a 1 μg dose of morphine in the PAG significantly increased latencies relative to vehicle treatment across doses ($F(3,25)= 23.79$, $p<.0001$), across times ($F(3,75)= 63.97$, $p<.0001$) and for the interaction between doses and times ($F(9,75)= 20.67$, $p<.0001$). The peak effect between the PAG and the DLP (Figure 6b), for example, was approximately half that observed with the same doses in the PAG/RVM combination (Figure 6a). Coadministration of different morphine doses (0.2-1 μg) in the PAG paired with a 1 μg dose of morphine in the DLP significantly increased latencies relative to

vehicle treatment across doses ($F(3,17)= 36.01$, $p<.0001$), across times ($F(3,51)= 21.89$, $p<.0001$) and for the interaction between doses and times ($F(9,51)= 40.61$, $p<.0001$). Simultaneous admininstration of 1 μg of morphine in the DLP significantly shifted ($F(2,34)= 5.21$, $p<.01$) the PAG dose-reponse curve to the left (Figure 5a), but not nearly as effectively as the RVM/PAG combination. A similar significant response ($F(2,47)= 12.61$, $p<.001$) was observed with the reciprocal study examining the DLP dose-reponse curve with a fixed PAG morphine dose (Figure 5b).

Coadministration of different morphine doses (0.3-1 μg) in the DLP paired with a 1 μg dose of morphine in the RVM significantly increased latencies relative to vehicle treatment across doses ($F(3,22)= 30.17$, $p<.0001$), across times ($F(3,66)= 32.41$, $p<.0001$) and for the interaction between doses and times ($F(9,66)= 16.49$, $p<.0001$). Coadministration of different morphine doses (0.2-1 μg) in the RVM paired with a 1 μg dose of morphine in the DLP significantly increased latencies relative to vehicle treatment across doses ($F(3,25)= 16.55$ $p<.0001$), across times ($F(3,75)= 57.76$, $p<.0001$) and for the interaction between doses and times ($F(9,75)= 11.64$, $p<.0001$). The coadministration of morphine into the DLP and the RVM yielded smaller analgesic interactions than combinations including the PAG, but the interactions remained greater than additive. This was seen both with peak effect and

duration of action (Figures 5b, 5c, 6b). Fixed doses of morphine in the RVM significantly shifted ($F(2,43)= 8.34$, $p<.001$) the DLP dose-reponse curve as effectively as fixed doses of morphine in the PAG (Figure 5b). However, fixed doses of morphine in the DLP significantly shifted ($F(2,54)= 4.17$, $p<.02$) the RVM dose-response curve to the left, but not as effectively as fixed doses of morphine in the PAG (Figure 5c).

These studies indicated that the strongest synergy existed between the PAG and the RVM. To examine whether these synergistic actions involved $mu_1$ receptors, rats were treated with naloxonazine. Significant differences in latencies were observed across treatments ($F(2,25)= 367.63$, $p<.0001$), across times ($F(3,75)= 18.09$, $p<.0001$) and for the interaction between treatments and times ($F(6,75)= 17.91$, $p<.0001$). Naloxonazine completely prevented antinociception elicited from the simultaneous morphine injections in the PAG and RVM (Figure 6a), implying that the synergy in these two regions involves $mu_1$ sites.

## DISCUSSION

Morphine is a potent antinociceptive agent when given either spinally or supraspinally, but it is most effective when administered to both sites simultaneously, the result of profound synergistic interactions (Pick, Roques, Gacel and Pasternak, 1992; Roerig and Fujimoto, 1989; Yeung and Rudy, 1980a; Pick et al., 1993). The present study found

that ineffective doses of morphine administered simultaneously into pairs of brainstem sites interact to produce significant antinociception. Given in both sites, morphine is almost an order of magnitude more potent than when given into either site alone. The efficacy of the antinociceptive interaction was observed both in terms of increasing peak antinociceptive responses as well as increasing duration of antinociceptive action. The most robust antinociceptive interactions were observed between the PAG and RVM; the DLP and RVM and the PAG and DLP pairs produced greater than additive responses. The PAG and RVM antinociceptive interaction was such that a small morphine dose in the PAG: a) transcended the cut-off score of 12 sec when morphine was administered into the RVM alone, and b) produced a 10-fold leftward shift in the RVM dose-response curve. Administration of consecutively higher doses of morphine in either the PAG or the DLP revealed increased latencies (10-12 sec) near cut-off scores. Whereas tail-flick latencies following morphine administration in the RVM (7-8 sec) never appoached cut-off scores. Therefore, when morphine was administered into the RVM alone, it produced a stepwise analgesic effect. The current studies establish the presence of synergy within the brainstem regions. The inclusion of fixed morphine doses in one region dramatically shifted the dose-response curve to the left in the other. Furthermore, a very low morphine dose in the PAG eliminates

the ceiling effect observed when morphine is given into the RVM alone.

These data strongly suggest that morphine has synergistic antinociceptive interactions within the brainstem in the same way that ventricular and intrathecal morphine antinociception displayed synergy (Yeung and Rudy, 1980a). Given concurrently, i.t. and i.c.v. morphine are almost an order of magnitude more potent than when given into either site alone. Synergy has also been observed for ventricular and intrathecal administration of the mu-selective opioid agonist, DAMGO (Malmberg and Yaksh, 1992). The present observations with intracerebral morphine also confirm prior studies using ethylketocyclazocine which inferred the presence of synergy between the PAG and the LC (Bodnar et al., 1991). In this study, ethylketocyclazocine proved to be a partial mu agonist incapable of producing antinociception in either region alone, but able to elicit a potent antinociception when coinjected into both areas simultaneously.

One argument against using the concept of synergy to explain the present results is that morphine diffused from one site to the other and produced additive effects. Several lines of evidence argue strongly against this nonspecific action. First, antinociception elicited from simultaneous administration of morphine into PAG (1 $\mu$g) and RVM (1 $\mu$g) sites was significantly greater than antinociception

elicited by a 2 μg dose of morphine into either the PAG alone or the RVM alone. Moreover, the duration of action in the interactive condition was more prolonged. Second, PAG/RVM interactions in antagonist studies are quite delimited. Microinjection into the RVM of either general, $5HT_2$, or $5HT_3$ antagonists as well as either general, mu or $delta_2$ opioid antagonists significantly reduce morphine antinociception elicited from the PAG (Kiefel et al., 1991,1992; Kiefel, Rossi and Bodnar, 1993). However, injections of peak doses of these serotonergic and opioid antagonists are ineffective in reducing mesencephalic morphine antinociception if the medullary cannulae are placed either lateral, dorsal or ventral to the RVM. Since the misplaced medullary cannula placements are far closer to the RVM than the PAG, this would also argue strongly against a diffusion hypothesis. Third, a simple diffusion hypothesis would predict that the largest effects should occur at the two sites which are closest to each other (e.g., PAG and DLP). In fact, these two sites produced the smallest synergistic interactions.

Thus, this study confirmed the presence of synergistic interactions between different supraspinal sites using the prototypical opiate, morphine. Insight into the pharmacological mechanisms of action mediating this synergistic interaction is only examined using the $mu_1$ antagonist, naloxonazine. As indicated previously, selective

agonists for mu (DAMGO), kappa (U50,488H), $delta_1$ (DPDPE), and $delta_2$ (deltorphin) receptors have been developed. To ascertain further the opioid receptor subtype(s) involved in this synergistic interaction, the last two experiments examined whether: a) these agonists produced intrinsic antinociception, and b) sub-antinociceptive doses of these agonists would produce synergy. The following experiments only used PAG-RVM pairs since the first experiment indicated that synergy was most efficacious in these sites.

**EXPERIMENT 2: Synergistic Brainstem Interactions for DAMGO Antinociception.**

Whereas the concept of multiple opiate receptors has been established (Lord et al., 1977; Schulz, Wuster, Kreuss and Herz, 1980; Frederickson, Smithwick, Shuman and Bemis, 1981; Ling and Pasternak, 1983; Satoh et al., 1983; Schmauss and Yaksh, 1984; Tung and Yaksh, 1982), it has been especially difficult to identify the involvement of a particular receptor subtype in a specific opioid effect (Hayes and Tyers, 1983; Porreca, Cowan, Raffa and Tallarida, 1982; Ward and Takemori, 1983). The demonstration of separate supraspinal opioid target sites (Herz et al., 1970; Pert and Yaksh, 1974; Wei, Sigel, Loh, and Way; 1975) has led to the suggestion that a specific effect may be mediated by different opioid receptors at different central nervous system sites (Ling and Pasternak, 1983; Porreca, Takemori, Sultana, Porteghese, Bowen and Mosberg, 1983; Stevens, Lacey, Miller, Elde and Seybold, 1991). In the previous experiment, morphine produced supraspinal antinociception and synergy. However morphine, the prototypic mu agonist (Martin et al., 1976), can act at other opioid receptor sites. Therefore, one cannot definitively conclude that supraspinal morphine antinociception and synergy elicited from the PAG and the RVM is the result of $\mu$ actions alone, or various combinations of mu-delta or mu-kappa receptors. Therefore, in this experiment, the following opioid agonists

with more selective actions were evaluated for their ability to elicit antinociception, and produce multiplicative interactions: DAMGO (mu), DPDPE (delta$_1$) and U50488H (kappa).

## METHODS

Single-Site Protocol: Rats were implanted with two cannulae, aimed at the PAG and the RVM. Each rat received a maximum of four single microinjection conditions at weekly intervals. Dose-response curves were assessed for DAMGO, U50,488H and DPDPE antinociception at each site (Table 3). All microinfusions were administered in 1 $\mu$l volumes, and vehicle, DAMGO, U50488H and DPDPE microinjections were in 0.9% normal saline. Tail-flick latencies were determined at 15, 30, 45 and 60 min following each microinjection. This time course of testing of the peptide analogues was chosen since the previous time course (60-120 min) was too long for the short-acting peptides. Therefore, the term "peak effect" in this and the following experiment is operationally defined as the 15 min time interval of tail-flick testing.

Double-Site Protocol: Rats were implanted with two cannulae aimed at either the PAG and the RVM. Each rat received a maximum of four double microinjection conditions at weekly intervals. The single-site protocol indicated that a 3 ng dose of DAMGO was relatively ineffective in producing significant antinociception from PAG and RVM placements

Table 3. Summary of DAMGO, U50488H and DPDPE microinjections into either the PAG alone or the RVM alone.

| Condition | Sample Size |
|---|---|
| A. PAG Placements. | |
| Vehicle | 18 |
| 1 ng DAMGO | 6 |
| 3 ng DAMGO | 8 |
| 10ng DAMGO | 7 |
| 20ng DAMGO | 7 |
| 20μg U50488H | 6 |
| 20μg DPDPE | 6 |
| B. RVM Placements. | |
| Vehicle | 18 |
| 3ng DAMGO | 8 |
| 10ng DAMGO | 7 |
| 20ng DAMGO | 6 |
| 20μg U50488H | 6 |
| 20μg DPDPE | 6 |

Note: PAG=Periaqueductal Gray; RVM=Rostro-ventral medulla; DLP=Dorsolateral Pons

alone. Thus, in the double-injection protocol, a 3 ng dose of DAMGO was administered into the first site of a pair, and a 3 ng dose of DAMGO administered into the second site of a pair simultaneously. Then, two-way dose-response curves were generated. In some animals, the 3 ng dose of DAMGO was held constant in the first site, and the dose of DAMGO administered into the second site was varied. In other animals, the 3 ng dose of DAMGO was held constant in the second site, and the dose of DAMGO administered into the first site was varied. Finally, this dose of DAMGO was administered into one site and a maximal dose of either U50,488H (20 μg) or DPDPE (20 μg) was administered into the second site. Table 4 summarizes the double-site injection paradigm for PAG-RVM placements. Tail-flick latencies were assessed 15, 30, 45 and 60 min after the double-site injections.

## RESULTS

**Histological Verification:** Mesencephalic cannula placements in the PAG group (Figure 7) were again localized in the lateral, ventral and ventro-lateral quadrants of the PAG and dorsal raphe nucleus as far rostral as the III cranial nerve nucleus and as far caudal as the dorsal raphe nucleus. Cannula placements in the RVM group (Figure 8) were localized in either the NRM, the NRGC or the NRGC, pars alpha as far rostral as the genu of the VII cranial nerve and as far caudal as the nucleus of the VII cranial nerve.

TABLE 4. Summary of the double-site microinjections of DAMGO, DPDPE and U50,488H in the PAG and RVM.

| PAG Condition | RVM Condition | Sample Size |
|---|---|---|
| Vehicle | Vehicle | 38 |
| A. DAMGO/DAMGO Combination. | | |
| DAMGO 3ng | DAMGO 3ng | 38 |
| DAMGO 3ng | DAMGO 1ng | 8 |
| DAMGO 3ng | DAMGO .5ng | 8 |
| DAMGO 1ng | DAMGO 3ng | 8 |
| B. DAMGO/U50488H Combination. | | |
| DAMGO 3ng | U50488H 20μg | 7 |
| U50488H 20μg | DAMGO 3ng | 7 |
| C. DAMGO/DPDPE Combination. | | |
| DAMGO 3ng | DPDPE 20μg | 7 |
| DPDPE 20μg | DAMGO 3ng | 7 |

---

Note: PAG=periaqueductal gray; RVM=rostral ventral medulla.

Figure 7. Histological verification of cannula placements using the atlas of Paxinos and Watson (1986). The figure depicts mesencephalic cannula placements (Bregma: -6.8 mm to -8.3 mm) in which DAMGO (10-20 ng), but not DPDPE nor U50488H, elicited a great analgesic response on the tail-flick test. Closed circles depict mesencephalic placements of rats within the PAG. Mesencephalic cannula placements in the PAG group were all localized in the lateral, ventral and ventro-lateral quadrants of the PAG and immediately-adjacent dorsal raphe nucleus. These placements were as far rostral as the III cranial nerve nucleus and as far caudal as the dorsal raphe nucleus. The numbers adjacent to the closed circles indicate the number of rats with cannula placements in that site, and the four numbers below each figure represent the corresponding plate number in the atlas of Paxinos and Watson.

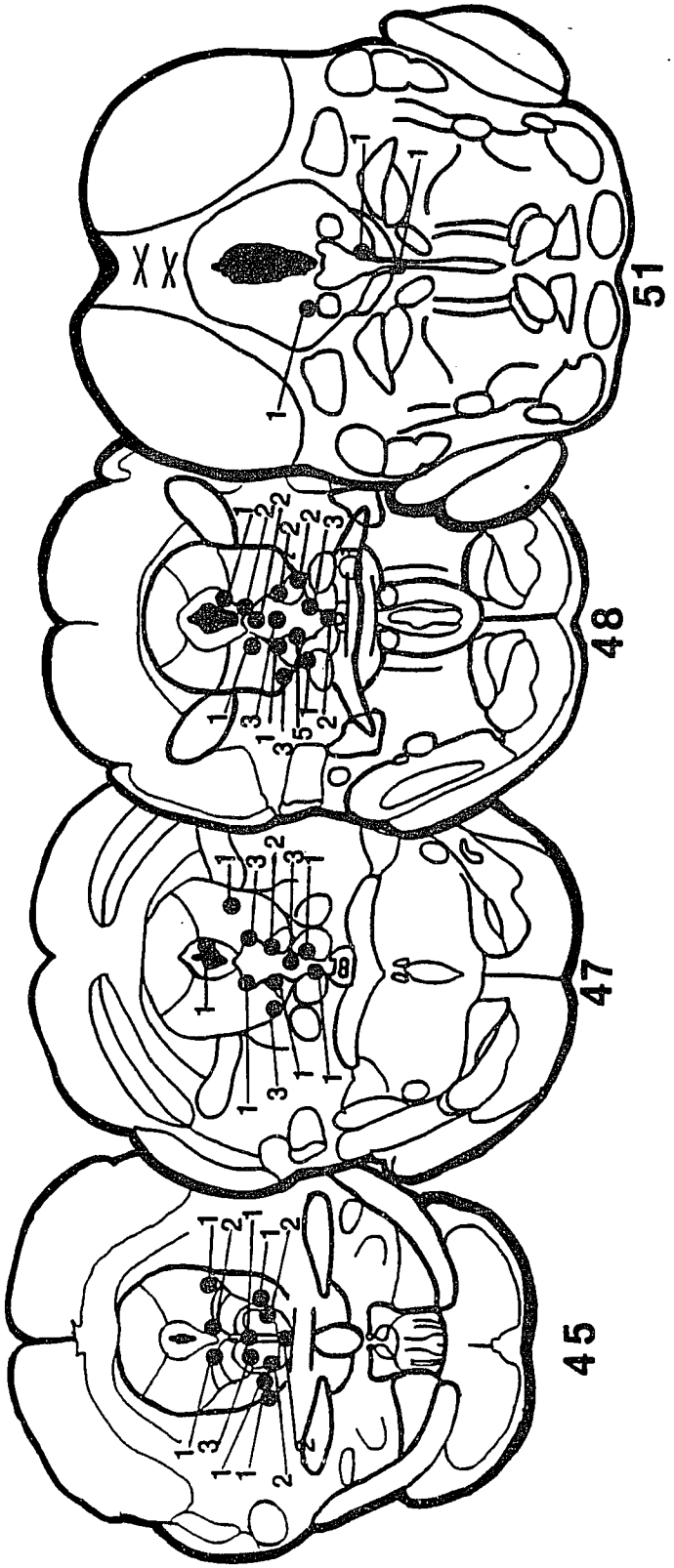
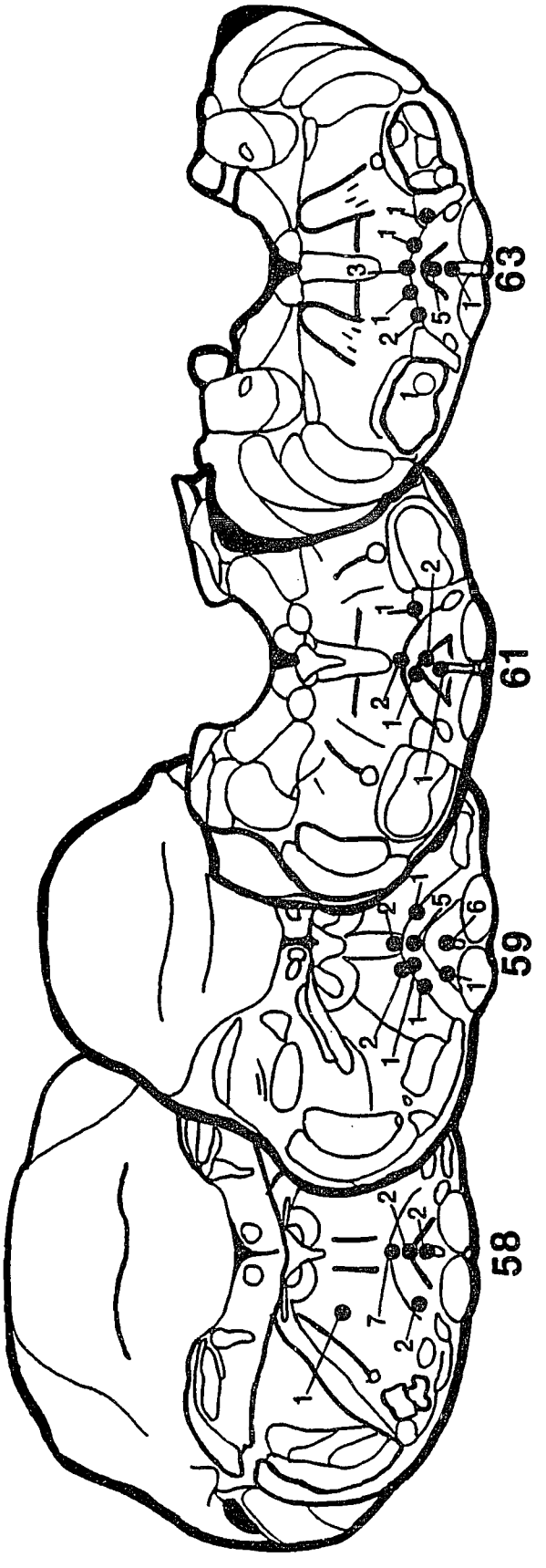

51
48
47
45

Figure 8. Histological verification of cannula placements using the atlas of Paxinos and Watson (1986). The figure depicts medullary cannula placements (Bregma: 10.04 mm to -11.3 mm) in which DAMGO (10-20 ng), but not DPDPE nor U50488H, elicited an analgesic response on the tail-flick test. The closed circles depict rats with medullary cannulae localized either in the NRM, NRGC or NRGC, pars alpha. These placements were as far rostral as the the genu of the VII cranial nerve and as far caudal as the nucleus of the VII cranial nerve. The numbers adjacent to the closed circles indicate the number of rats with cannula placements in that site, and the four numbers below the figure represent the corresponding plate number in the atlas of Paxinos and Watson.


63
61
59
58

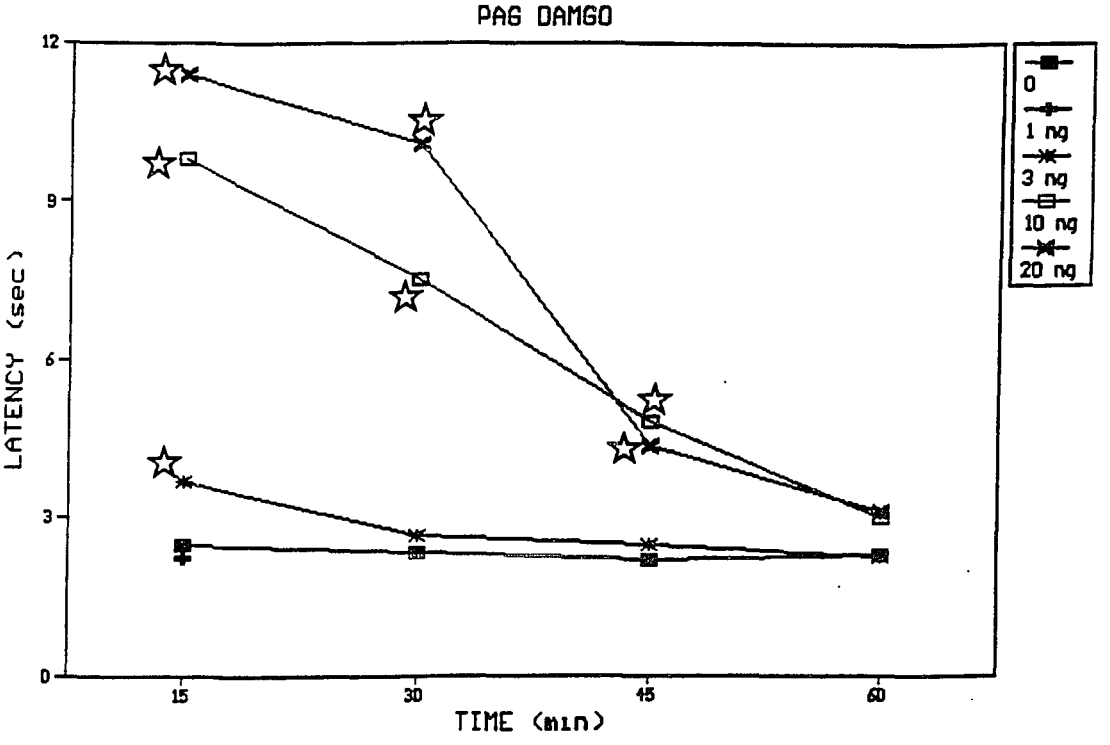
**Single-Site Injections:** For PAG placements, significant differences in tail-flick latencies were observed among DAMGO doses ($F(4,39)= 75.60$, $p<.0001$), across times ($F(3,117)= 184.06$, $p<.0001$) and for the interaction between doses and times ($F(12,117)= 53.99$, $p<.0001$). DAMGO in the PAG significantly elevated latencies in a dose-dependent manner with a peak effect at 15 min and a duration of action of approximately 45 min (Figure 9a). For RVM placements, significant differences in tail-flick latencies were observed among DAMGO doses ($F(3,33)= 36.33$, $p<.0001$), across times ($F(3,99)= 121.39$, $p<.0001$) and for the interaction between doses and times ($F(9,99)= 35.53$, $p<.0001$). DAMGO in the RVM significantly elevated latencies in a dose-dependent manner with a peak effect at 15 min and a duration of action of approximately 45 min (Figure 9b). These dose-response curves indicate that the latencies observed following a 3 ng dose of DAMGO in the PAG or RVM are minimally altered.

In contrast, administration of either DPDPE or U50488H into either the PAG or RVM failed to alter latencies relative to vehicle treatment across conditions ($F(4,37)= 0.34$), across times ($F(3,111)= 2.30$) or for the interaction between conditions and times ($F(12,111)= 0.92$) (Figures 10a and 10b).

**Regional Interactions for DAMGO**

Coadministration of a fixed DAMGO dose (3 ng) in one site paired with variable (0.5-3 ng) doses of DAMGO in a

Figure 9. Time Action Curves of Single-Site DAMGO Injections. Rats cannulated in the a) PAG or b) RVM received the stated DAMGO injections (1.0, 3.0, 10, 20 ng). Results in figure 9a. indicate mean-tail-flick latencies over a 15, 30, 45 and 60 min time course. Significant increases in tail-flick latencies were observed across DAMGO 3.0, 10 and 20 ng doses, across time courses (15, 30, 45 min), and for the interactions between doses and times. Open stars indicate latencies significantly different from vehicle injections. Results in figure 9b. indicate mean-tail-flick latencies over 15, 30, 45 and 60 min time course. Significant increases in tail-flick latencies were observed across DAMGO 10 and 20 ng doses, across time courses (15, 30, 45 min), and for the interactions between doses and times. Open stars indicate latencies significantly different from vehicle injections.


PAG DAMGO
LATENCY (sec)
TIME (min)
0
1 ng
3 ng
10 ng
20 ng

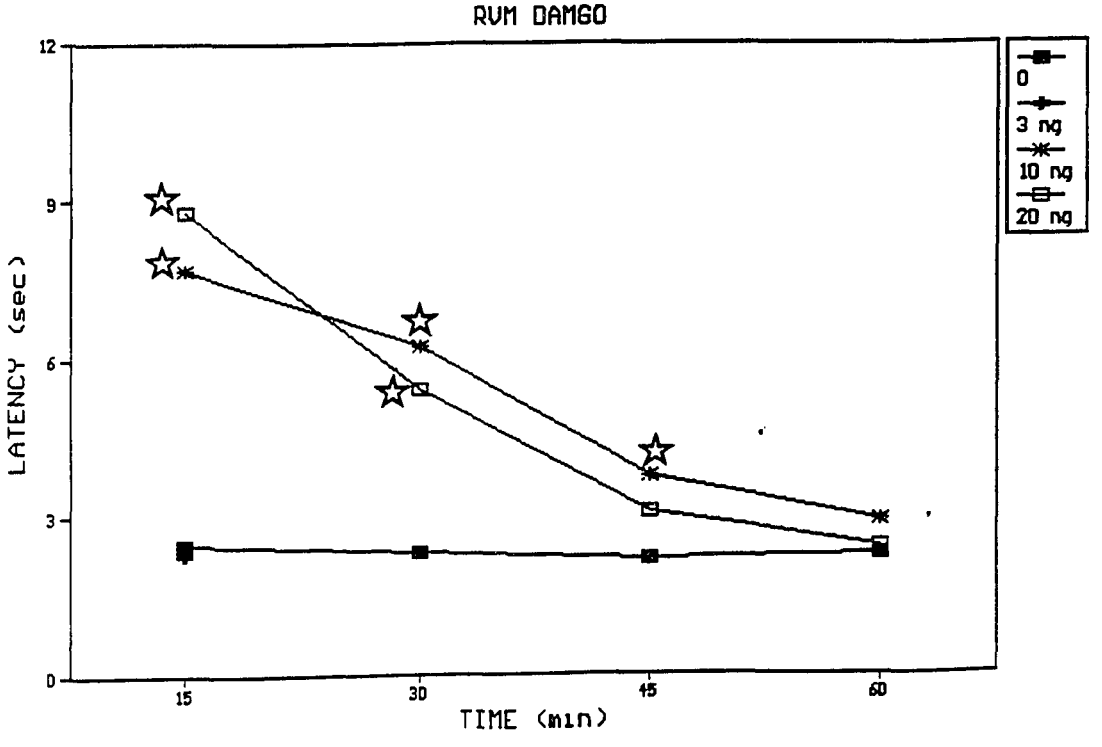
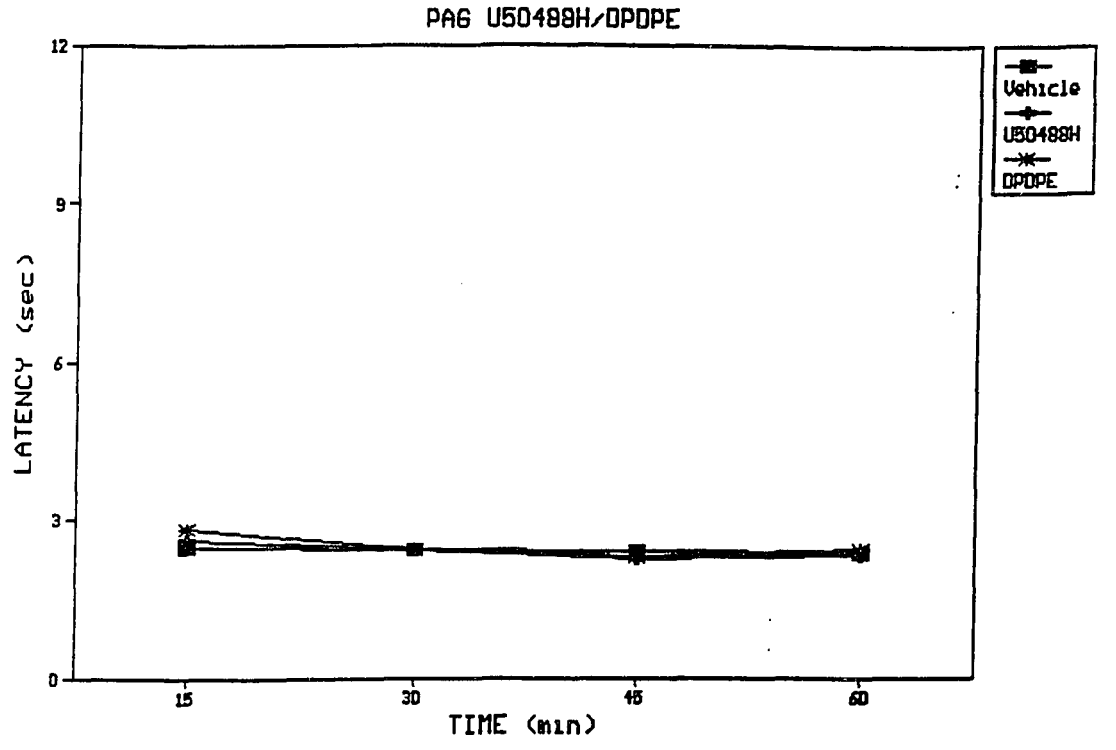
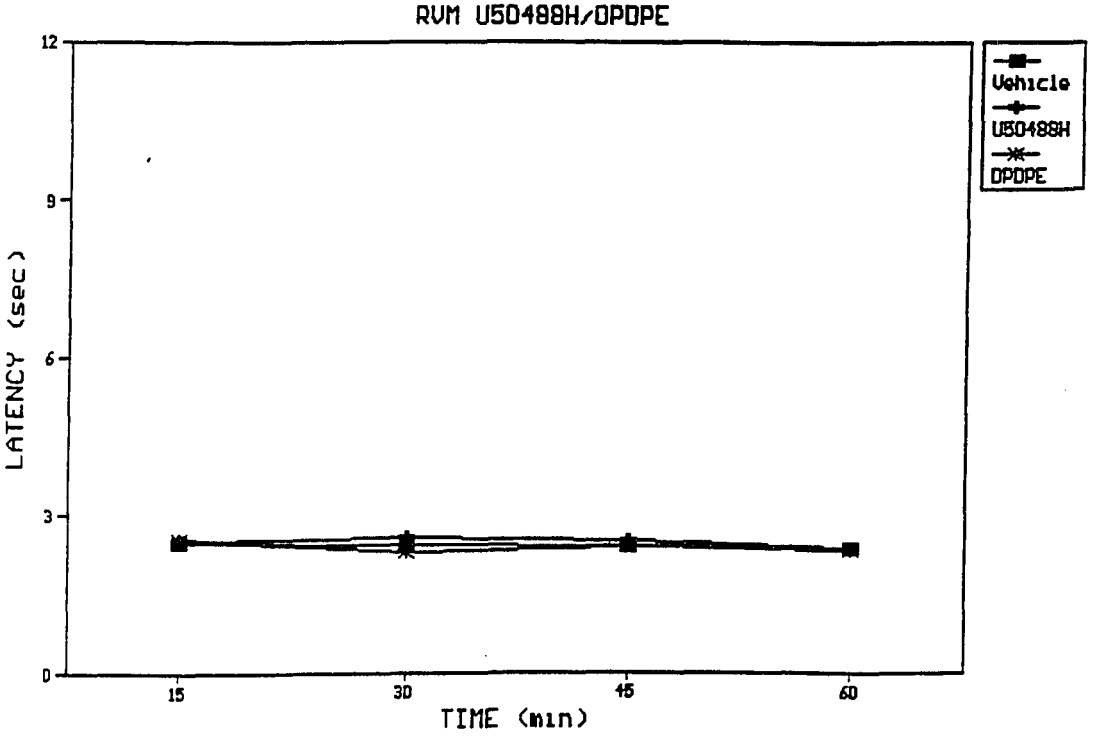

RVM DAMGO
LATENCY (sec)
TIME (min)
0
3 ng
10 ng
20 ng

Figure 10. Time Action Curves of Single-Site U50488H and DPDPE Injections. Rats cannulated in the 10a) PAG or 10b) RVM received microinjections of either 10 and/or 20 μg of U50488H or DPDPE. Results indicate mean-tail-flick latencies. Significant increases in tail-flick latencies were not observed across U50488H or DPDPE doses, across time courses, and not for the interactions between doses and times.

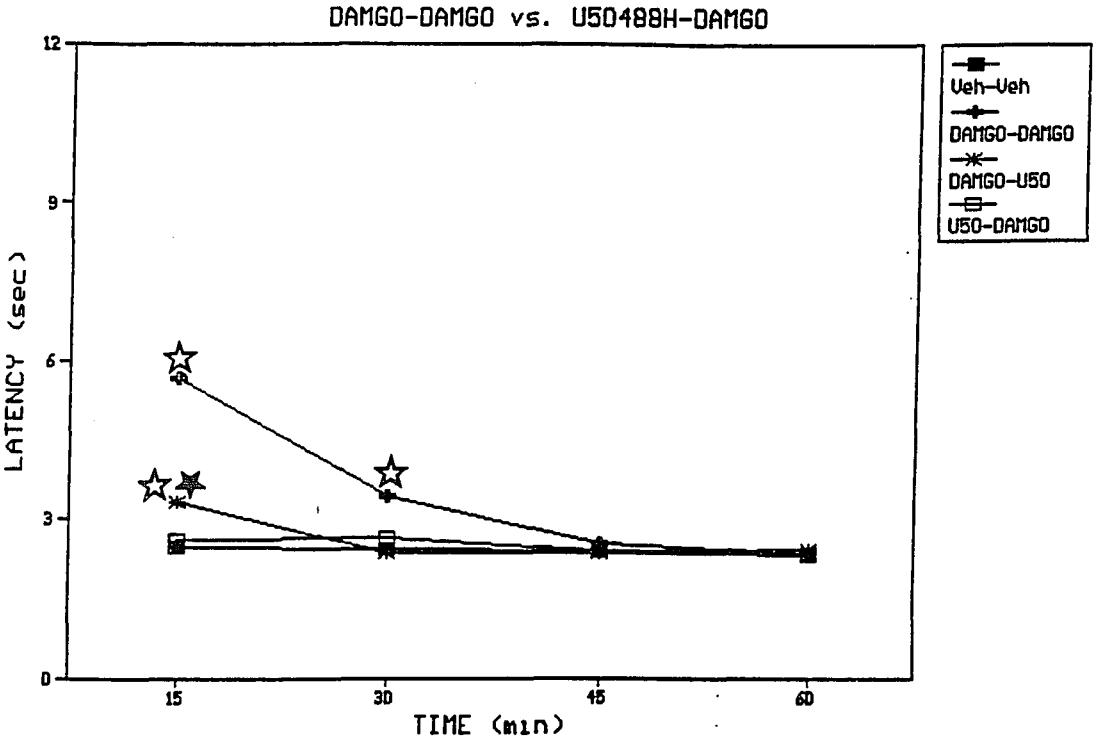

PAG U50488H/OPDPE
LATENCY (sec)
12
9
6
3
0
15
30
45
60
TIME (min)
Vehicle
U50488H
OPDPE


RVM U50488H/OPDPE
LATENCY (sec)
12
9
6
3
0
15
30
45
60
TIME (min)
Vehicle
U50488H
OPDPE

second site significantly increased latencies relative to vehicle treatment across doses ($F(4,109)= 58.30$, $p<.0001$), across times ($F(3,327)= 94.75$, $p<.0001$) and for the interaction between doses and times ($F(12,327)= 53.78$, $p<.0001$). Coadministration of DAMGO (3 ng) into both the PAG and RVM significantly increased latencies for up to 30 min (Figure 11). Administration of a fixed DAMGO dose of 3 ng in the PAG significantly shifted the dose-response curve for DAMGO in the RVM to the left by approximately 6-fold (Figure 12a). Administration of a fixed DAMGO dose of 3 ng in the RVM produced smaller (2-fold) leftward shifts in the dose-response curve for DAMGO in the PAG (Figure 12b).

Comparisons of the efficacy of double-site microinjections of DAMGO-DAMGO, DAMGO-U50488H and DAMGO-DPDPE revealed significant differences among conditions ($F(5,112)= 46.52$, $p<.0001$), across times ($F(3,336)= 47.42$, $p<.0001$) and for the interaction between conditions and times ($F(15,336)= 43.28$, $p<.0001$). In contrast to the significant increases in latencies following DAMGO (3 ng) in the PAG and DAMGO (3 ng) in the RVM, administration of U50,488H (20 $\mu$g) in the PAG and DAMGO (3 ng) in the RVM failed to alter latencies (Figure 11a). However, when DAMGO (3 ng) was administered in the PAG and U50,488H (20 $\mu$g) was administered in the RVM, latencies were significantly increased 15 min following coadministration (Figure 11a). However, this effect was significantly less than the DAMGO-

Figure 11. Time Action Curves of a Fixed DAMGO dose (3 ng) in the PAG and the RVM as Compared to Coadministration of a Fixed DAMGO dose (3 ng) in One Site Paired with Variable doses of either U50488H or DPDPE in a second site. Results in figure 11a. indicate mean-tail-flick latencies. Small significant increases in tail-flick latencies were observed only across DAMGO 3 ng in the PAG / U50488H 20 μg dose in the RVM, and only across the 15 min time course. Open stars indicate latencies significantly different from DAMGO (3 ng) in PAG and DAMGO (3 ng) in the RVM. Closed stars indicate latencies significantly different from vehicle injections. Results in figure 11b indicate mean-tail-flick latencies. Small significant increases in tail-flick latencies were observed only across DAMGO 3ng in the PAG / DPDPE 20 μg dose in the RVM, and only across the 15 min time course. Open stars indicate latencies significantly different from DAMGO (3 ng) in PAG and DAMGO (3 ng) in the RVM. Closed stars indicate latencies significantly different from vehicle injections.


DAMGO-DAMGO vs. U50488H-DAMGO
LATENCY (sec)
TIME (min)
Veh-Veh
DAMGO-DAMGO
DAMGO-U50
U50-DAMGO

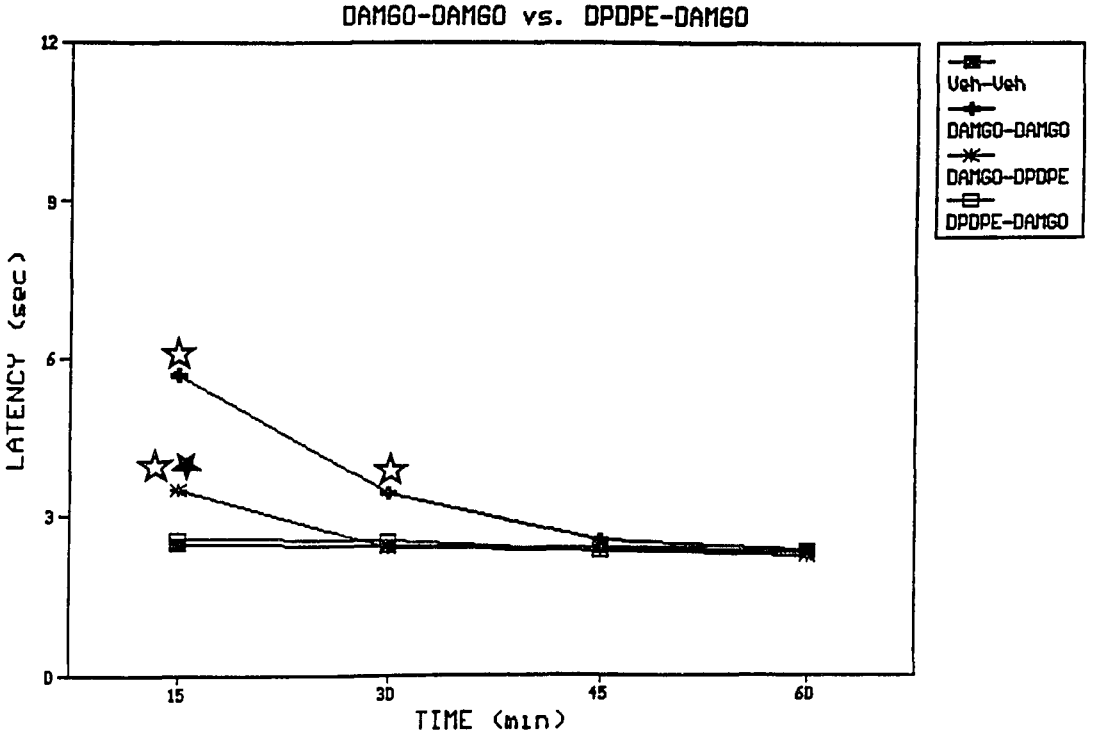
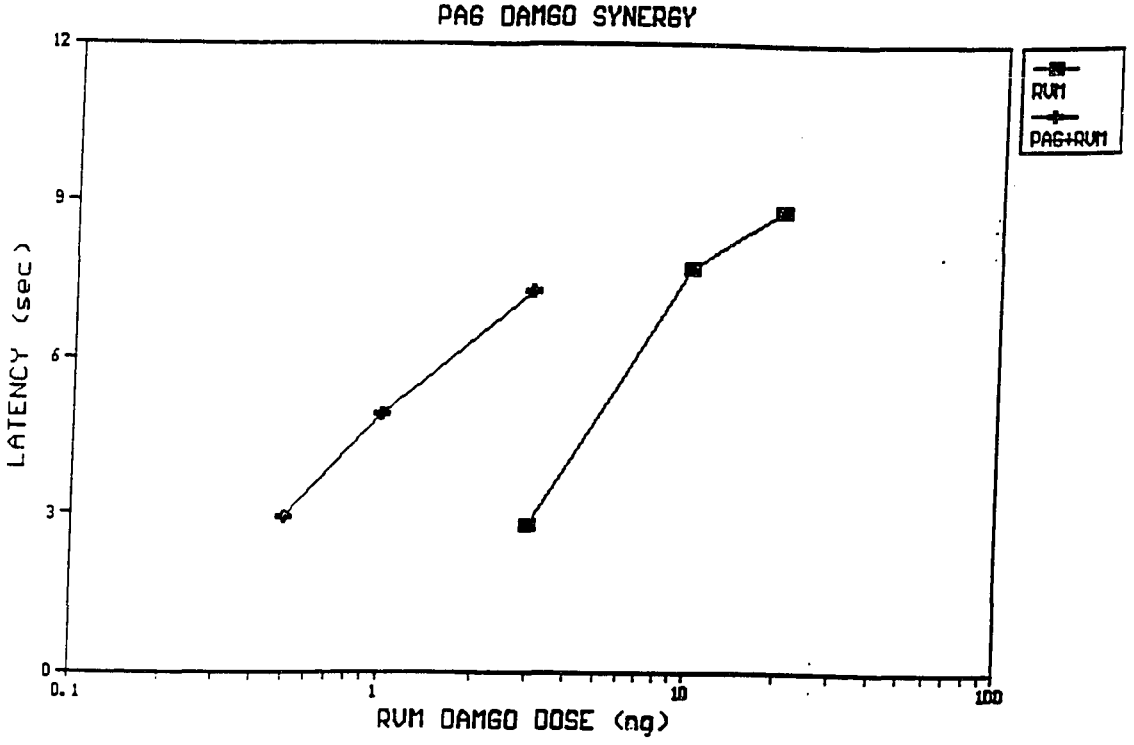
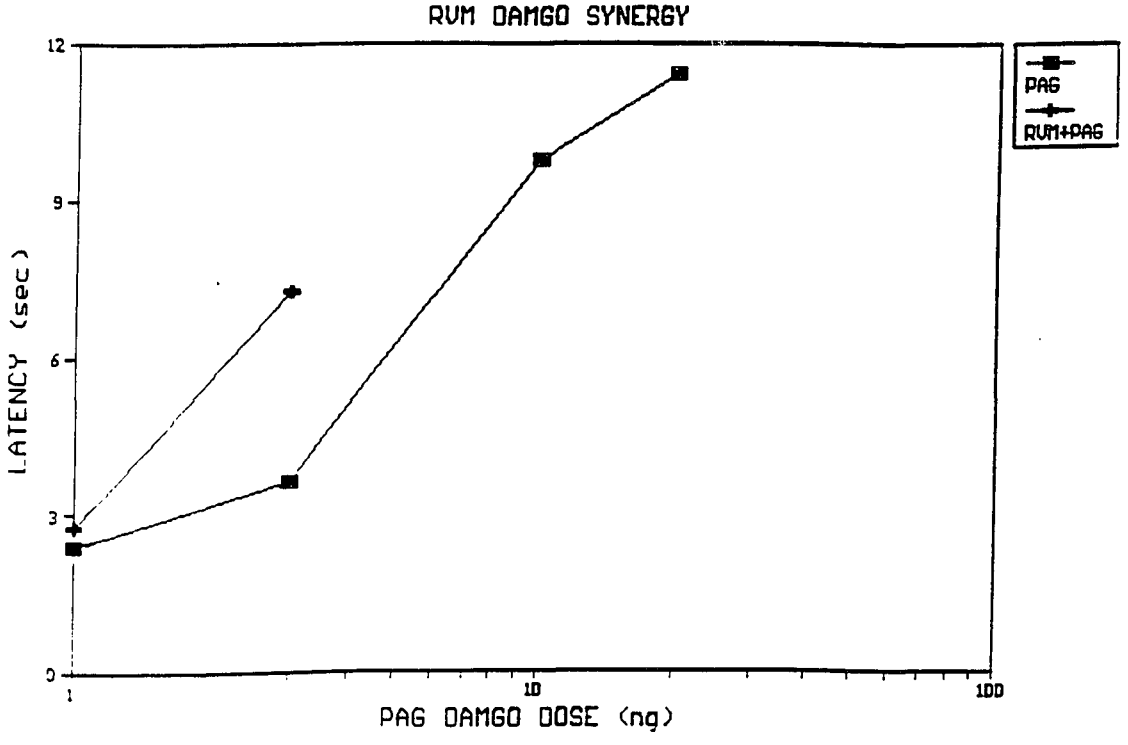

DAMGO-DAMGO vs. DPDPE-DAMGO
LATENCY (sec)
TIME (min)
Veh-Veh
DAMGO-DAMGO
DAMGO-DPDPE
DPDPE-DAMGO

Figure 12. DAMGO Dose-Response Curves in Individual Sites and in Combinations. Cannulated rats received a fixed dose of DAMGO (3 ng) in the PAG and a variable dose (0.5-1 ng) in the RVM, and a second group of rats received a fixed dose of DAMGO (3ng) in the RVM and a dose of 1 ng in the PAG. Results indicated that the administration of a fixed DAMGO dose of 3 ng in the PAG significantly shifted the dose-response curve for DAMGO in the RVM to the left by approximately 6-fold (figure 12a). The mean peak tail-flick latencies in figure 12a depicts the addition of DAMGO (1 ng) in the RVM produced significant increases across PAG doses, across the time course (15 and 30 min), and significant increases for the interactions between doses and times. The administration of a fixed DAMGO dose of 3 ng in the RVM produced smaller (2-fold) leftward shifts in the dose-response curve for DAMGO in the PAG (figure 12b). This fixed RVM dose significantly increased latencies when paired with the 0.7 and 1 μg PAG dose, but not the 0.3 μg dose.


PAG DAMGO SYNERGY
LATENCY (sec)
12
9
6
3
0
0.1
1
10
100
RVM DAMGO DOSE (ng)
RVM
PAG+RVM


RVM DAMGO SYNERGY
LATENCY (sec)
12
9
6
3
0
1
10
100
PAG DAMGO DOSE (ng)
PAG
RVM+PAG

DAMGO treatment, and was comparable to the small increases in latencies induced by DAMGO (3 ng) applied to the PAG itself (Figure 9a). The same pattern of effects occurred for DPDPE (20 μg: Figure 11b). It failed to increase latencies when applied to the PAG and paired with DAMGO (3 ng) in the RVM. It increased latencies for 15 min when applied to the RVM and paired with DAMGO (3 ng) in the PAG. Again, this effect was significantly less than the DAMGO-DAMGO treatment, and was comparable to the small increases in latencies induced by DAMGO (3 ng) applied to the PAG itself (Figure 9a).

## DISCUSSION

The first experiment demonstrated that morphine produced dose-dependent antinociception following intracerebral administration into the PAG, RVM and DLP, and that simultaneous administration of sub-antinociceptive morphine doses into pairs of these sites produced a multiplicative interaction. Since naloxonazine blocked the development of synergy between PAG and RVM placements, this implied a role for $mu_1$ receptors in this effect. The second experiment characterized opioid receptor subtype involvement in multiplicative interactions between the PAG and RVM directly through the use of selective opioid receptor subtype agonists for mu (DAMGO), kappa (U50488H) and $delta_1$ (DPDPE) receptors.

Whereas nanogram doses of DAMGO produced a significant

antinociceptive response from PAG and RVM placements, microgram doses of either U50488H or DPDPE failed to alter latencies from either site alone. These data extend and confirm previous studies indicating that activation of mu, but not $delta_1$ or kappa, receptors elicits supraspinal, intracerebral opioid antinociception (e.g., Bodnar et al., 1988, 1991; Drower, Stapelfeld, Ragerty, DeCosta, Rice and Hammond, 1991; Fang et al., 1986; Malmberg and Yaksh, 1992; Smith et al., 1988). When sub-antinociceptive doses of DAMGO were simultaneously administered into the PAG and RVM, a potent and more prolonged antinociception occurred, indicating the presence of a multiplicative interaction. A fixed dose of DAMGO in the PAG produced significant leftward (6-fold) shifts in the RVM dose-response curve for DAMGO which was more pronounced than the 2-fold leftward shift in the PAG dose-response curve for DAMGO following a fixed dose of DAMGO in the RVM. These data closely parallel the more efficacious multiplicative interactions observed for morphine in PAG and RVM placements. Therefore, administration of selective mu agonists produce the same pattern of multiplicative effects as morphine, providing a second, independent line of evidence for this receptor subtype in mediating supraspinal synergy.

The failure of DPDPE and U50488H to produce antinociception in the PAG and RVM argues against $delta_1$ and kappa involvement in these sites. However, there is a

possibility that delta$_1$ and kappa receptors in either the PAG or RVM might interact with mu receptors to produce multiplicative interactions. In investigating this possibility, the present study found that this was clearly not the case. Simultaneous administration of low DAMGO doses in one site paired with high doses of U50488H or DPDPE in a second site failed to meaningfully alter latencies. Thus, the mu-mediated supraspinal synergistic interactions do not appear to rely upon kappa or delta$_1$ receptors for their expression.

**EXPERIMENT 3: Synergistic Brainstem Interactions for Deltorphin Antinociception.**

Recent work has indicated the existence of two novel, delta-selective opioid agonists. Porreca and colleagues (1992) have indicated that the selective delta$_1$ receptor agonist, DPDPE (Sofuoglu, Portoghese and Takemori, 1991), and the highly-selective delta$_2$ agonist, deltorphin II, have been shown to produce antinociception following i.c.v. or i.t. administration (Jiang et al., 1991; Sofuoglu et al., 1991; Mattia et al., 1991). However, whereas DPDPE antinociception is blocked by the delta$_1$ antagonist, DALCE, but not delta$_2$-selctive analogues of naltrindole, deltorphin antinociception is blocked by delta$_2$-selective analogues of naltrindole, but not the delta$_1$ antagonist, DALCE. Finally, DPDPE and deltorphin fail to produce cross-tolerance following intrathecal administration.

The second experiment indicated that delta$_1$-selective agonists failed to elicit antinociception or synergy from PAG and RVM placements. Given the dissociative effects of DPDPE and deltorphin, it is conceivable that delta$_2$ agonists may exert different supraspinal effects. Evidence for this is provided by the observation that naltrindole itself administered into the RVM can block mesencephalic morphine antinociception in a manner similar to that of the general opioid antagonist, naltrexone, and the mu-selective antagonist, BFNA (Kiefel et al., 1993). Although naltrindole

itself is a general delta antagonist, the failure of $delta_1$ agonists suggests a potential role for $delta_2$ sites. Thus, the third experiment determined the relative contribution of the $delta_2$ opioid receptor subtype in antinociceptive and synergistic processes using deltorphin to establish: a) whether antinociception is elicited from PAG and RVM sites, b) whether sub-antinociceptive doses of deltorphin administered simultaneously to the two sites produce multiplicative interactions, and c) whether deltorphin paired with either the mu-selective agonist, DAMGO or the $delta_1$-selective agonist, DPDPE would produce multiplicative interactions following simultaneous administration.

## METHODS

Single-Site Protocol: Rats were implanted with two cannulae aimed at the PAG and the RVM. Each rat received a maximum of four single microinjection conditions at weekly intervals. Dose-response curves were assessed for deltorphin antinociception at each site (Table 5). All microinfusions were administered in 1 $\mu$l volumes, and vehicle and deltorphin microinjections were in distilled water and 0.1% glacial acetic acid. Tail-flick latencies were determined at 15, 30, 45 and 60 min following each microinjection.

Double-Site Protocol: Rats were implanted with two cannulae aimed at the the PAG and the RVM. Each rat received a maximum of four double microinjection conditions at weekly

Table 5. Summary of deltorphin microinjections into either the PAG alone, the RVM alone or simultaneously into the PAG and the RVM.

| Condition | Sample Size |
|---|---|
| A. PAG Alone. | |
| Vehicle | 16 |
| 10 μg | 5 |
| 20 μg | 8 |
| B. RVM Alone. | |
| Vehicle | 16 |
| 10 μg | 5 |
| 20 μg | 6 |

C. Double-Site Injections

| PAG | RVM | Sample Size |
|---|---|---|
| Vehicle | Vehicle | 50 |
| Delt 10μg | Delt 10μg | 6 |
| Delt 10μg | DAMGO 3ng | 6 |
| DAMGO 3ng | Delt 10μg | 6 |
| Delt 10μg | DAMGO .5μg | 3 |
| DAMGO .5ng | Delt 10μg | 6 |
| Delt 10μg | DAMGO 1ng | 6 |
| DAMGO 1ng | Delt 10μg | 6 |
| Delt 5μg | DAMGO 3ng | 3 |
| Delt 7.5μg | DAMGO 3ng | 6 |
| DAMGO 3ng | Delt 7.5μg | 6 |
| Delt 10μg | DPDPE 20μg | 6 |
| DPDPE 20μg | Delt 10μg | 6 |

---

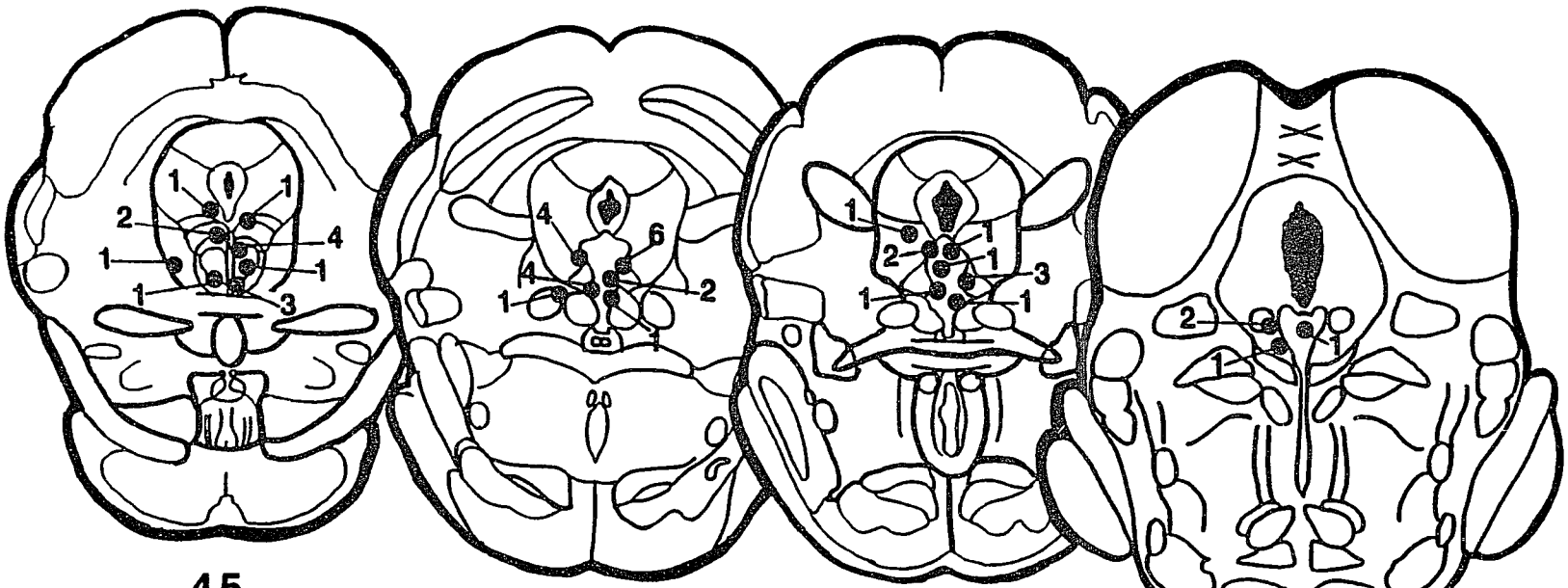
Note: PAG=periaqueductal gray; RVM=rostral ventral medulla; Delt=deltorphin II.

intervals. The single-site protocol indicated that a 10 μg dose of deltorphin was ineffective in producing significant antinociception from PAG and RVM placements alone. Thus, in the double-injection protocol, a 10 μg dose of deltorphin was administered into the first site of a pair, and a 10 μg dose of deltorphin administered into the second site of a pair simultaneously. Then, two-way dose-response curves were generated for deltorphin and DAMGO. In some animals, either a 10 μg dose of deltorphin or a 3 ng dose of DAMGO was held constant in the first site, and the dose of DAMGO or deltorphin administered into the second site was varied. In other animals, either a 10 μg dose of deltorphin or a 3 ng dose of DAMGO was held constant in the second site, and the dose of DAMGO and deltorphin administered into the first site was varied. Finally, the 10 μg dose of deltorphin was administered into one site and a maximal dose of DPDPE (20 μg) was administered into the second site. Table 5 summarizes the double-site injection paradigm for PAG-RVM placements. Tail-flick latencies were assessed 15, 30, 45 and 60 min after the double-site injections.

## RESULTS

**Histological Verification:** Mesencephalic cannula placements in the PAG group (Figure 13) were again localized in the lateral, ventral and ventro-lateral quadrants of the PAG and dorsal raphe nucleus as far rostral as the III cranial nerve nucleus and as far caudal as the dorsal raphe nucleus.

Figure 13. Histological verification of cannula placements using the atlas of Paxinos and Watson (1986). The figure depicts mesencephalic cannula placements (Bregma: -6.8 mm to -7.64 mm) in which deltorphin (20 μg), elicited an analgesic response on the tail-flick test. Closed circles depict mesencephalic placements of rats within the PAG. Mesencephalic cannula placements in the PAG group were all localized in the lateral, ventral and ventro-lateral quadrants of the PAG and immediately-adjacent dorsal raphe nucleus. These placements were as far rostral as the III cranial nerve nucleus and as far caudal as the dorsal raphe nucleus. The numbers adjacent to the closed circles indicate the number of rats with cannula placements in that site, and the four numbers below each figure represent the corresponding plate number in the atlas of Paxinos and Watson.


45
47
48
51

Cannula placements in the RVM group (Figure 14) were localized in either the NRM, the NRGC or the NRGC, pars alpha as far rostral as the genu of the VII cranial nerve and as far caudal as the nucleus of the VII cranial nerve.

**Single-Site Injections:** For PAG and RVM placements, significant differences in tail-flick latencies were observed among deltorphin doses ($F(4,35)= 19.71$, $p<.0001$), across times ($F(3,105)= 118.35$, $p<.0001$) and for the interaction between doses and times ($F(12,105)= 24.77$, $p<.0001$). Deltorphin significantly elevated latencies in the PAG and RVM in a dose-dependent manner with a peak effect at 15 min and a duration of action of approximately 30 min (Figures 15a and 15b). These dose-response curves indicate that the latencies observed following a 10 μg dose of deltorphin in the PAG or RVM are minimally altered.

**Regional Interactions for Deltorphin**

Coadministration of a fixed deltorphin dose (10 μg) in one site paired with fixed doses of either deltorphin (10 μg) or DPDPE (20 μg) in a second site significantly increased latencies relative to vehicle treatment across times ($F(3,60)= 17.33$, $p<.0001$) and for the interaction between conditions and times ($F(9,60)= 3.04$, $p<.005$), but not across conditions ($F(3,20)= 1.47$). Coadministration of deltorphin (10 μg) into both the PAG and RVM significantly, but minimally increased latencies for only 15 min (Figure 16). If DPDPE was paired with deltorphin in either pair of

Figure 14. Histological verification of cannula placements using the atlas of Paxinos and Watson (1986). The figure depicts medullary cannula placements (Bregma: 10.04 mm to -11.3 mm) in which deltorphin (20 ng) elicited an analgesic response on the tail-flick test. The closed circles depict rats with medullary cannulae localized either in the NRM, NRGC or NRGC, pars alpha. These placements were as far rostral as the the genu of the VII cranial nerve and as far caudal as the nucleus of the VII cranial nerve. The numbers adjacent to the closed circles indicate the number of rats with cannula placements in that site, and the four numbers below the figure represent the corresponding plate number in the atlas of Paxinos and Watson.

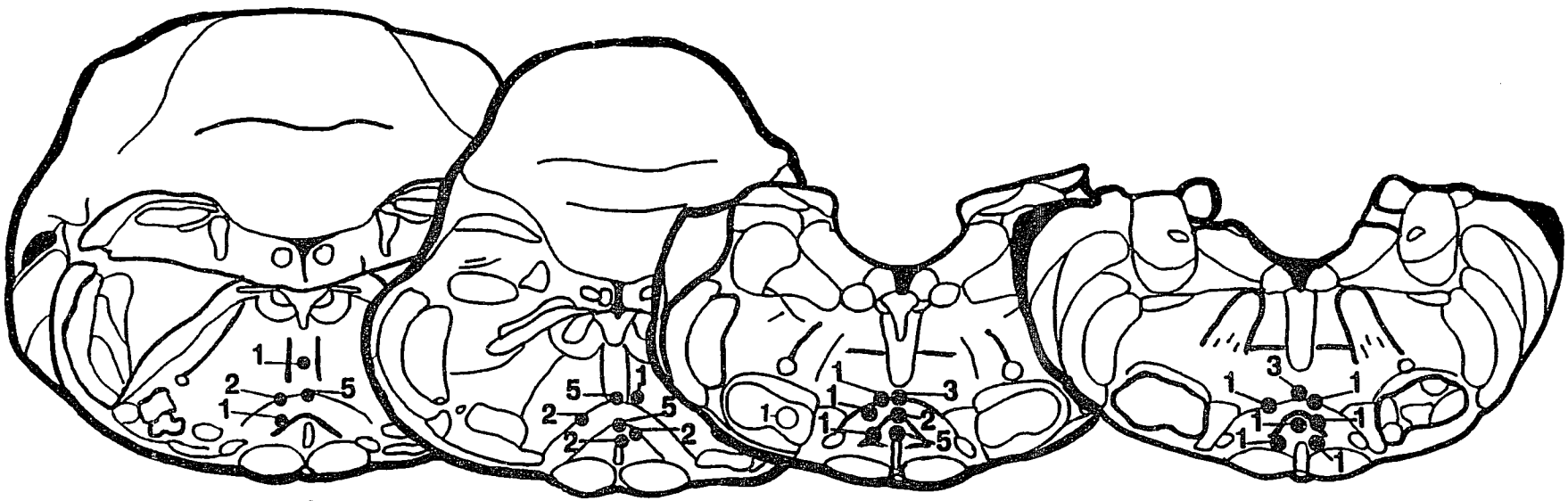
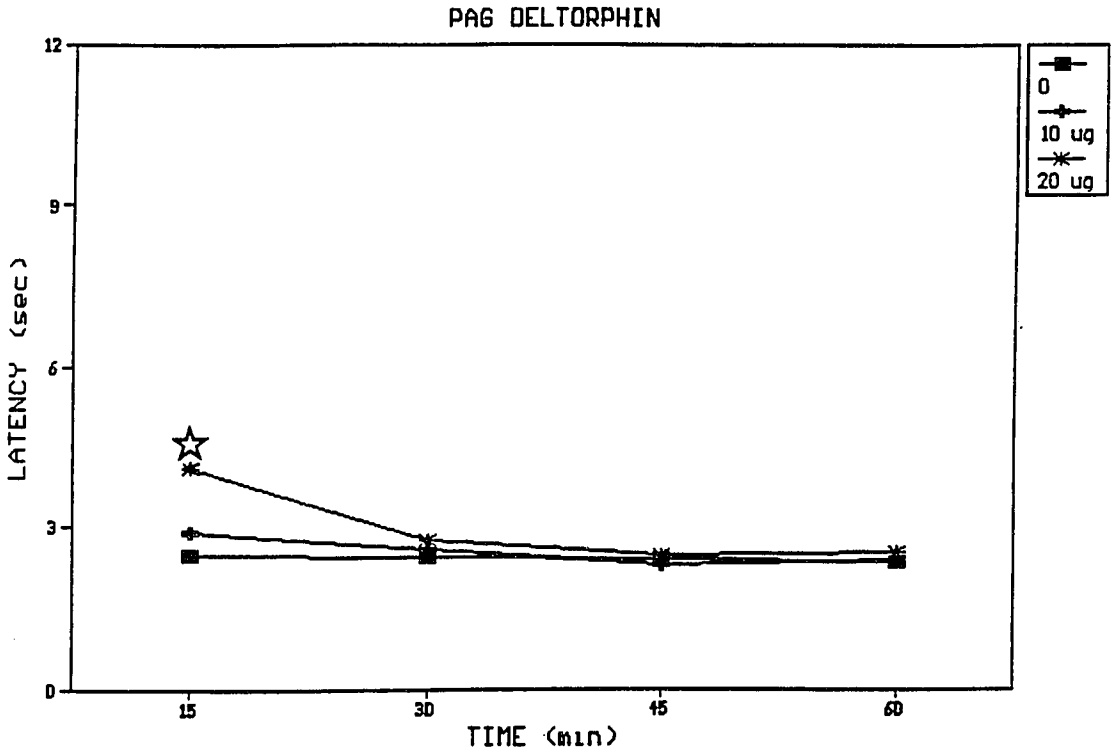
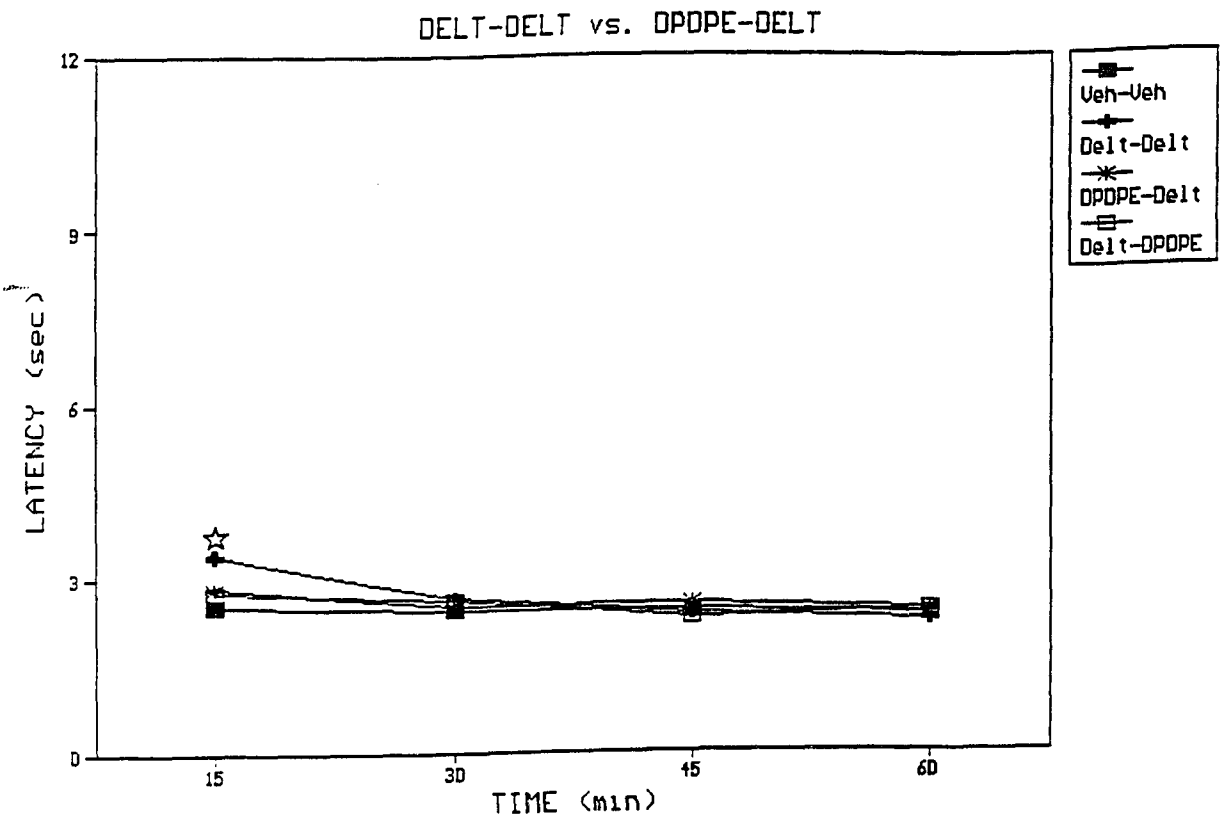

58
59
61
63

Figure 15. Time Action Curves of Single-Site Deltorphin Injections. Rats cannulated in the a) PAG or b) RVM received the stated deltorphin injections (10, 20 μg). Results in figures 15a. and 15b indicate mean-tail-flick latencies over a 15, 30, 45 and 60 min time course. Significant increases in tail-flick latencies in fig 15a were observed across deltorphin 10 and 20 μg doses, across the 15 min time course, and for the interactions between doses and times. Open stars indicate latencies significantly different from vehicle injections. Significant increases in tail-flick latencies in fig 15b were observed across deltorphin 10 and 20 μg doses, across time courses (15, 30 min), and for the interactions between doses and times. Open stars indicate latencies significantly different from vehicle injections. These dose-response curves indicate that the latencies observed following a 10 μg dose of deltorphin in the PAG or RVM are minimally altered.


PAG DELTORPHIN
LATENCY (sec)
12
9
6
3
0
15
30
45
60
TIME (min)
0
10 ug
20 ug


RVM DELTORPHIN
LATENCY (sec)
12
9
6
3
0
15
30
45
60
TIME (min)
0
10 ug
20 ug

Figure 16. Time Action Curves of a Fixed Deltorphin dose (10 μg) in the PAG and the RVM as Compared to Coadministration of a Fixed Deltorphin dose (10 μg) in One Site Paired with a DPDPE (20 μg) dose in the Second Site. Results in figure 16 indicate mean-tail-flick latencies across a 15, 30, 45 and 60 min time course. Coadministration of deltorphin (10 μg) into both the PAG and RVM significantly, but minimally increased latencies for only 15 min. If DPDPE was paired with deltorphin in either pair of sites, this combination failed to alter latencies.


DELT-DELT vs. DPDPE-DELT
LATENCY (sec)
12
9
6
3
0
15
30
45
60
TIME (min)
Veh-Veh
Delt-Delt
DPDPE-Delt
Delt-DPDPE

sites, this combination failed to alter latencies.

Coadministration of DAMGO (3 ng) into either the PAG or RVM with varied doses (5-10 μg) of deltorphin in the other site produced significant differences in latencies relative to vehicle treatment across conditions ($F(7,133)= 78.77$, $p<.0001$), across times ($F(3,399)= 75.12$, $p<.0001$) and for the interaction between conditions and times ($F(21,399)= 28.72$, $p<.0001$). The synergistic interactions observed for both deltorphin (10 μg) in the PAG and DAMGO (3 ng) in the RVM and DAMGO (3 ng) in the PAG and deltorphin (10 μg) in the RVM were similar in magnitude and duration to that observed for DAMGO (3 ng) in the PAG and DAMGO (3 ng) in the RVM (Table 6). Administration of DAMGO (3 ng) in the PAG produced leftward shifts in the deltorphin dose-response curve in the RVM (Figure 17a). Administration of DAMGO (3 ng) in the RVM also produced leftward shifts in the deltorphin dose-response curve in the PAG (Figure 17b).

Coadministration of deltorphin (10 μg) into either the PAG or RVM with varied doses (0.5-3 ng) of DAMGO in the other site produced significant differences in latencies relative to vehicle treatment across conditions ($F(7,133)= 85.94$, $p<.0001$), across times ($F(3,399)= 76.33$, $p<.0001$) and for the interaction between conditions and times ($F(21,399)= 32.23$, $p<.0001$). Administration of deltorphin (10 μg) in the PAG produced leftward shifts in the DAMGO dose-response curve in the RVM (Figure 18a). Administration of deltorphin

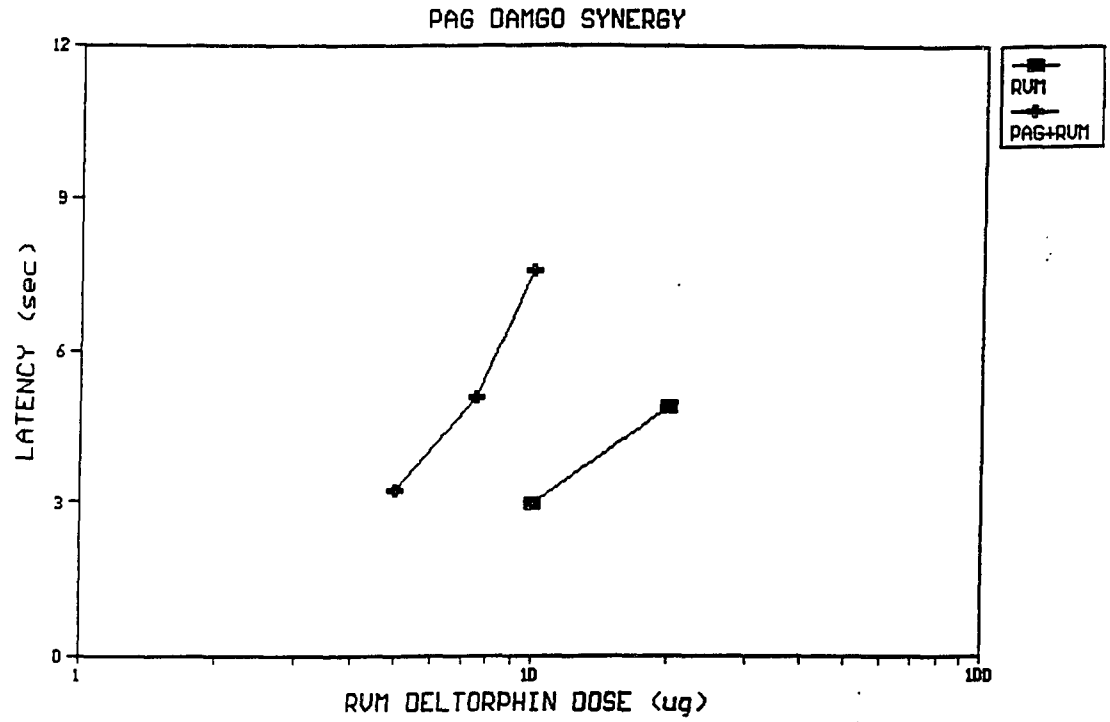
TABLE 6. Comparison of DAMGO-DAMGO and Deltorphin-DAMGO Synergistic Interactions.

| PAG | RVM | Post-Injection (min) | | | |
|---|---|---|---|---|---|
| | | 15 | 30 | 45 | 60 |
| Vehicle | Vehicle | 2.47 | 2.40 | 2.43 | 2.33 |
| DAMGO (3ng) | DAMGO (3ng) | 6.00* | 3.35* | 2.53 | 2.38 |
| Delt (10μg) | DAMGO (3ng) | 6.15* | 5.99* | 5.44* | *4.64 |
| DAMGO (3ng) | Delt (10μg) | 7.56* | 4.75* | 4.93* | *4.11 |

---

Note: Delt=Deltorphin
*=significantly different from mean vehicle scores.

Figure 17. DAMGO-Deltorphin Dose-Response Curves in Individual Sites and in Combinations. Cannulated rats received a fixed dose of DAMGO (3 ng) in the PAG and a variable dose of deltorphin (5.0, 7.5, 10 μg) in the RVM, and a second group of rats received a fixed dose of DAMGO (3ng) in the RVM and a deltorphin dose of 5.0, 7.5, 10 μg in the PAG. Results indicated that coadministration of DAMGO (3 ng) into either the PAG or RVM with varied doses (5-10 μg) of deltorphin in the other site produced significant differences in latencies relative to vehicle treatment. The synergistic interactions observed for both deltorphin (10 μg) in the PAG and DAMGO (3 ng) in the RVM and DAMGO (3 ng) in the PAG and deltorphin (10 μg) in the RVM were similar in magnitude and duration to that observed for DAMGO (3 ng) in the PAG and DAMGO (3 ng) in the RVM. Administration of DAMGO (3 ng) in the PAG produced leftward shifts in the deltorphin dose-response curve in the RVM (Figure 17a). Administration of DAMGO (3 ng) in the RVM also produced leftward shifts in the deltorphin dose-response curve in the PAG (Figure 17b).


PAG DAMGO SYNERGY
12
9
6
3
0
LATENCY (sec)
1
10
100
RVM DELTORPHIN DOSE (ug)
RVM
PAG+RVM

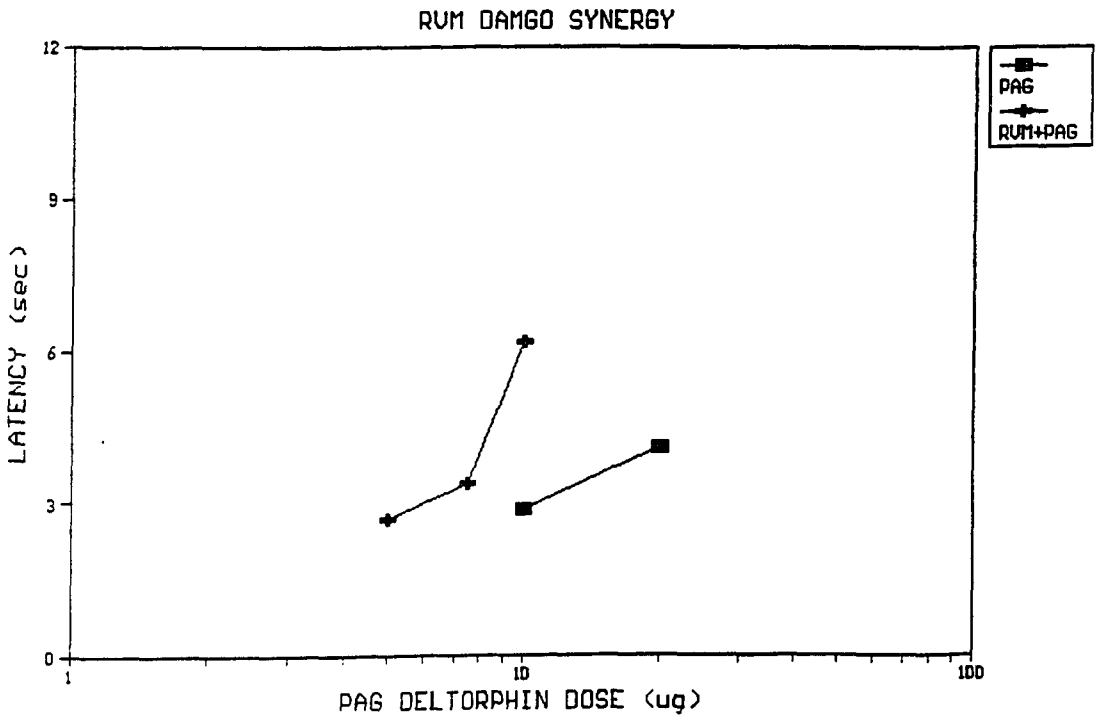
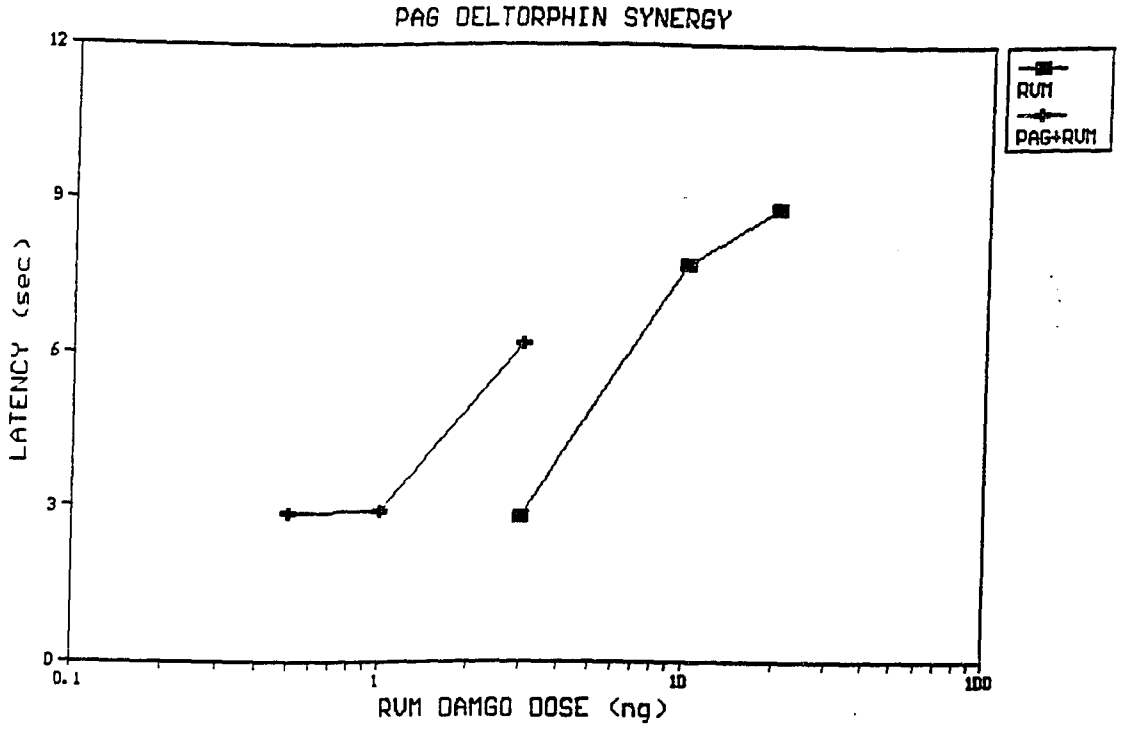
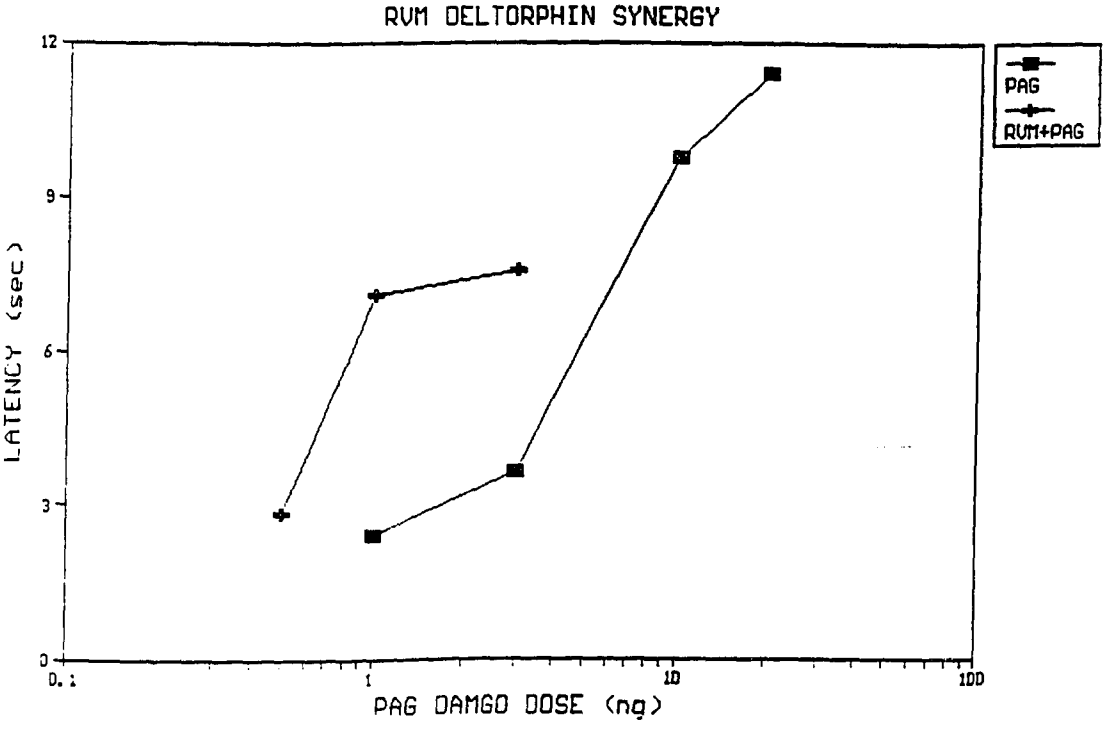

RVM DAMGO SYNERGY
12
9
6
3
0
LATENCY (sec)
1
10
100
PAG DELTORPHIN DOSE (ug)
PAG
RVM+PAG

Figure 18. Deltorphin-DAMGO Dose-Response Curves in Individual Sites and in Combinations. Cannulated rats received a fixed dose of deltorphin (10 μg) in the PAG and a variable dose of DAMGO (0.5, 1.0, 3.0 ng) in the RVM, and a second group of rats received a fixed dose of deltorphin (10 μg) in the RVM and a DAMGO dose of 0.5, 1.0 and 3.0 ng in the PAG. Results indicated that coadministration of coadministration of deltorphin (10 μg) into either the PAG or RVM with varied doses (0.5-3 ng) of DAMGO in the other site produced significant differences in latencies relative to vehicle treatment ($p<.0001$). Administration of deltorphin (10 μg) in the PAG produced leftward shifts in the DAMGO dose-response curve in the RVM (figure 18a). Administration of deltorphin (10 μg) in the RVM also produced leftward shifts in the DAMGO dose-response curve in the PAG (figure 18b).


PAG DELTORPHIN SYNERGY
LATENCY (sec)
12
9
6
3
0
0.1
1
10
100
RVM DAMGO DOSE (ng)
RVM
PAG+RVM


RVM DELTORPHIN SYNERGY
LATENCY (sec)
12
9
6
3
0
0.1
1
10
100
PAG DAMGO DOSE (ng)
PAG
RVM+PAG

(10 μg) in the RVM also produced leftward shifts in the DAMGO dose-response curve in the PAG (Figure 18b).

## DISCUSSION

Deltorphin produced mild, but significant increases in tail-flick latencies following microinjection into the PAG and RVM. These increases were more potent than similar doses of either DPDPE or U50488H administered into the same sites, supporting previous studies (Porecca et al., 1992; Raffa, Martinez and Porecca, 1992; Stefano, Melchiorri, Negri, Hughes and Scharrer, 1992). However, deltorphin was far less potent than either morphine or DAMGO in eliciting antinociception from PAG or RVM sites. Simultaneous coadministration of deltorphin (10 μg) into both the PAG and RVM produced a small, but significant increase in latencies that lasted for only 15 min. These effects are in stark contrast to the highly potent and longer-duration effects observed following simultaneous administration of sub-antinociceptive doses of either morphine or DAMGO into the PAG and RVM. Further, while fixed doses of either morphine or DAMGO in one site produced significant leftward shifts in its corresponding dose-response curve in the second site, deltorphin failed to produce such potent shifts. However, the effects of simultaneous administration of deltorphin into this pair of sites were more efficacious than the simultaneous administration of the $delta_1$ agonist, DPDPE into one site, and the $delta_2$ agonist, deltorphin into the

second site. This lack of any multiplicative effect provides further support for the view that these different delta subtypes are separable. (Porreca et al., 1992; Jiang et al., 1991a; Sofuoglu et al., 1991a; Mattia et al., 1991) or i.t. administration (Sofuoglu et al., 1991; Mattia et al., 1992).

In contrast, when sub-antinociceptive doses of deltorphin were paired with sub-antinociceptive doses of DAMGO in PAG and RVM placements, multiplicative interactions occurred that were of both greater potency and longer duration. Indeed, the pairing of deltorphin and DAMGO was quite similar in pattern to the pairing of DAMGO and DAMGO and the pairing of morphine and morphine. The maximal antinociception elicited from deltorphin-DAMGO pairings was comparable to that of DAMGO and DAMGO. Second, there was an increase in the duration of antinociception elicited from deltorphin and DAMGO pairs. Finally, administration of a fixed dose of deltorphin to one site produced significant leftward shifts in DAMGO's dose-response curve in the second site. Similarly, administration of a fixed dose of DAMGO to one site produced significant leftward shifts in deltorphin's dose-response curve in the second site. These data lead to two potential conclusions: a) that distinct $delta_2$ and mu receptor systems exist, and that they interact with each other; and b) that the $delta_2$ and mu receptor agonists are exerting their interactive effects through a common receptor subtype, and that this receptor subtype is

the $mu_1$ site.

The first conclusion is supported by the previously-cited evidence that deltorphin produces antinociception through a distinct and selective receptor subtype. It is also supported by the observation that naltrindole administered into the RVM can block morphine antinociception elicited from the PAG (Kiefel et al., 1993). However, some questions remain about the biochemical specificty of $delta_2$ agonists and antagonists. While they have been clearly dissociated biochemically and physiologically from $delta_1$ sites, it is not clear whether they interact with other opioid sites. The $mu_1$ site has historically demonstrated high affinity for a wide range of opioid ligands, including morphine, ethylketocyclazocine, beta-endorphin, DADL and DSLET (see review: Pasternak and Wood, 1986; Wolozin and Pasternak, 1981; Fang et al., 1986; Hahn et al, 1982). In contrast, other opioid ligands, including DPDPE and U50488H show little affinity for the $mu_1$ site (Clark et al., 1989; Takahashi, Senda, Kaneto, 1991; Roerig and Fujimoto, 1989). There have not been published studies indicating whether the $delta_2$ agonist, deltorphin or $delta_2$ antagonists, including naltrindole and its analogues, show affinity for $mu_1$ binding. If they do, it would be important to characterize the affinity of this binding relative to DAMGO so as to establish the interaction between DAMGO and deltorphin. A second approach towards delineating between the two cited

conclusions is to examine whether antinociception elicited by DAMGO and deltorphin in the PAG and RVM are similarly affected by the delta antagonist, naltrindole and the $mu_1$ antagonist, naloxonazine. Studies to this end are in progress.

## GENERAL DISCUSSION

The observed antinociceptive interactions between PAG and RVM sites, between DLP and RVM sites and between PAG and DLP sites have implications in understanding the underlying anatomical, physiological and pharmacological substrates of supraspinal opiate analgesia. The antinociceptive interactions between the PAG and RVM appear to be subserved by direct connections between the PAG and the NRM (Abols and Basbaum, 1981; Beitz et al., 1983; Carlton, Leichnetz, Young and Mayer, 1983; Gallagher and Pert, 1978) and between the PAG and the NRGC (Beitz et al., 1983; Fardin, Oliveras and Besson, 1984; Van Bockstaele et al., 1991; Van Bockstaele et al., 1989). Immunocytochemical analyses have indicated the presence of serotonergic and enkephalinergic fibers in this PAG/RVM pathway (Beitz, 1982a; Beitz 1982b). The antinociceptive interactions between the RVM and DLP are supported by anatomical interconnections between the NRGC and the DLP (Clark and Proudfit, 1991) and the NRGC and the DLP (Aston-Jones et al., 1986; Ennis and Aston-Jones, 1987). Indeed some NRGC neurons send collateralized projections to the DLP and PAG (Van Bockstaele and Aston-Jones, 1992). Recent studies have indicated that the connections between the NRGC and LC are predominantly enkephalinergic and adrenergic in that order (Drolet, Van Bockstaele and Aston-Jones, 1992; Pieribone and Aston-Jones, 1983). Finally, the antinociceptive interactions between the DLP and PAG are

supported by anatomical and physiological evidence (Ennis et al., 1991).

The antinociceptive interactions between PAG and RVM sites complements ongoing physiological research in which two types of RVM cells ("on-cells" and "off-cells") appear to modulate nociceptive responses and predict the antinociceptive effects of systemic morphine (Fields et al., 1991). Whereas "off-cells" are activated by systemic morphine (Fields et al., 1983), "on-cells" are inhibited by systemic morphine (Barbaro et al., 1986). Administration of morphine in the PAG inhibits glutamate-induced activation of "on-cells" in the RVM (Morgan and Liebeskind, 1987), and iontophoretic application of morphine depresses "on-cell", but not "off-cell" activity in the RVM (Heinricher, Morgan and Fields, 1992). It would appear that the most pronounced modulation of medullary "on-cells" and "off-cells" originates in the PAG, which parallels the present findings that the PAG/RVM antinociceptive interaction was the most pronounced in terms of peak effects, duration of action and shifts in each site's dose-response curve. Research combining pharmacological interactions with physiological activity may clarify the mechanisms of synergistic action.

The antinociceptive interactions between these pairs of sites provides further clarification of pharmacological analyses of supraspinal morphine antinociception. Substantial evidence supports the view that morphine

antinociception elicited from single supraspinal sites is mediated through mu receptors (Fang et al., 1986; Jensen and Yaksh, 1986c; Smith et al., 1988; Bodnar et al., 1988, Bodnar, 1990), which have been divided into two subclasses, $mu_1$ and $mu_2$, based upon both pharmacological and biochemical studies (Lutz, Cruciani, Munson and Rodbard, 1985; Lutz, Cruciani, Costa, Munson and Rodbard,1984; Pasternak and Wood, 1986; Pick et al., 1992; Pick, Paul and Pasternak, 1991; Rothman, Jacobson, Rice and Herkenham, 1987; Rothman, Long, Bykov, Jacobson, Rice and Holaday, 1988; Toll, Keys, Plogar and Loew, 1984; Ling and Pasternak, 1983). The $mu_1$ receptor has been implicated in the mediation of opiate antinociception following intracerebroventricular administration (Ling and Pasternak, 1983; Ling, Simantov, Clark and Pasternak, 1986; Paul et al., 1989) and following intracerebral administration into the PAG, DLP, and RVM (Bodnar et al., 1988, 1990). On the other hand, $mu_2$ receptors appear to control the mu-mediated actions upon spinal antinociception (Pick et al., 1992; Pick et al., 1993) as well as the brainstem mu receptors involved with supraspinal/spinal synergism (Roerig and Fujimoto, 1989). Ventricular administration of low doses of TRIMU-5, a $mu_2$ agonist/$mu_1$ antagonist, (Tive, Paul, Gacel and Pasternak, 1992) antagonizes ventricular morphine antinociception yet enhances intrathecal morphine antinociception, providing further evidence of dissociable supraspinal $mu_1$ and spinal

$mu_2$ effects. The sensitivity of the synergism between the PAG and the RVM to naloxonazine in the current study implicates a $mu_1$ mechanism of action for supraspinal sites. This complements the recent finding (Kiefel et al., 1993) that administration of the mu-selective antagonist, beta-funaltrexamine, into the RVM virtually eliminates morphine analgesia elicited from the PAG.

In view of the fact that nanogram doses of DAMGO produced a significant antinociceptive response from PAG and RVM placements, and that microgram doses of either U50488H or DPDPE failed to alter latencies from either site alone, these data confirm and extend previous studies indicating that activation of mu, but not $delta_1$ or kappa, receptors elicited supraspinal, intracerebral opioid antinociception. When sub-antinociceptive doses of DAMGO were simultaneously administered into the PAG and RVM, a potent and more prolonged antinociception occurred, indicating the presence of a multiplicative interaction. These data closely parallel the more efficacious multiplicative interactions observed for morphine microinjections in PAG and RVM. Additionally, deltorphin produced small antinociception and a small multiplicative effect within the PAG and the RVM. This appeared to be modulated by $delta_2$ receptors, since the $delta_1$ agonist, DPDPE failed to produce antinociception or interact with other agonists to produce synergy. Moreover, deltorphin and DAMGO produced multiplicative interactions

similar to simultaneous morphine administration into both the PAG and RVM, and simultaneous DAMGO administration into both sites. Also, the administration of DPDPE and U50488H alone, and in conjunction with simultaneous administration of low DAMGO doses, failed to produce antinociception in the two sites, arguing against $delta_1$ and kappa invovement in these sites. Thus, this provided further evidence for the $mu_1$ receptor subtype in mediating supraspinal synergy.

Perhaps the most convincing evidence implicating brainstem $mu_2$ receptors in spinal/supraspinal synergy comes from studies with inbred CXBK mice (Pick et al., 1993). CXBK mice have a deficiency of $mu_1$ sites with relatively normal levels of $mu_2$ binding (Moskowitz and Goodman, 1985a). Pharmacologically, CXBK mice do not display analgesia to morphine given supraspinally, a mu1 action, but retain a normal sensitivity towards intrathecal morphine, a $mu_2$ action (Moskowitz and Goodman, 1985b; Vaught, Mathiasen, Raffa, 1988; Pick et al., 1993). In a spinal/supraspinal model, the sensitivity of CXBK mice to intracerebroventricular morphine is normal (Pick et al., 1993). Thus, $mu_1$ receptors do not appear to play a role in spinal/supraspinal synergy. Thus, the ability to distinguish pharmacologically between intrinsic brainstem synergy and spinal/supraspial synergy may prove to be an important tool in future investigations.

In conclusion, the brainstem contains an intrinsic

synergistic system that is distinct from the previously described spinal/supraspinal system (Pick et al., 1992, 1993; Roerig and Fujimoto, 1989; Yeung and Rudy, 1980a). and this system includes the PAG, the DLP and the RVM. All three of these sites receive nociceptive inputs which are differentially sensitive to different levels of noxious input. Although interactions appear to involve all three regions, they are most pronounced between the PAG and the RVM.

# GENERAL CONCLUSIONS

The present dissertation examined supraspinal antinociceptive synergy and opioid receptor subtype agonists, and the following conclusions were made:

1. Morphine produced powerful synergistic interactions within the PAG, the RVM and the DLP.
2. Synergy occurred in the DLP-RVM and the DLP-PAG, however the most robust response was seen when morphine was simultaneously injected into the PAG and the RVM.
3. Naloxonazine completely prevented antinociception from simultaneous morphine injections, implying that the synergy in these regions involved $mu_1$ sites.
4. DAMGO produced powerful antinociceptive responses in the PAG and the RVM, and synergy similar to that of morphine.
5. The synergistic interaction seen in these sites did not involve $kappa_1$ nor $delta_1$ receptors, as shown by the failure of U50488H and DPDPE to produce antinociception.
6. Deltorphin produced small antinociception and a small multiplicative effect within the PAG and the RVM. This appeared to be modulated by $delta_2$ receptors, since the $delta_1$ agonist, DPDPE failed to produce antinociception **or** interact with other agonists to produce synergy.
7. Deltorphin and DAMGO produce multiplicative interactions similar to simultaneous morphine administration into both the PAG and RVM, and simultaneous DAMGO

administration into both the PAG and RVM. The synergistic interactions in this dissertation lead to the conclusion that distinct $delta_2$ and mu receptor systems exist, and that they interact with each other. Additionally, the $delta_2$ and mu receptor agonists are exerting their interactive effects through a common receptor subtype, and this receptor subtype is the $mu_1$ site.

8. In conclusion, supraspinal synergy occurred between different sites in the brain, arguing for an interacting and potentially integrating system, dedicated to pain control and pain inhibition. Activation, through pharmacological or neurosurgical manipulations of such critical supraspinal areas, have eventual implications for the treatment of chronic pain and other dysfunctional pain states in humans.

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