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Mesencephalic morphine antinociception: Antagonism by serotonergic and opioid antagonists in the rostral ventral medulla in rats

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Mesencephalic Morphine Antinociception: Antagonism by Serotonergic and Opioid Antagonists in the Rostral Ventral Medulla in Rats

by

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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.

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Abstract

Mesencephalic Morphine Antinociception: Antagonism by Serotonergic and Opioid Antagonists in the Rostral Ventral Medulla in Rats

by

Jacqueline M. Kiefel

Adviser: Dr. Richard J. Bodnar

Supraspinal opioid antinociception is mediated in part by connections between the midbrain periaqueductal gray (PAG) and the rostral ventral medulla (RVM), which includes the nuclei raphe magnus and reticularis gigantocellularis. Little is known about the nature of the neurochemical link between the PAG and the RVM. Since both serotonergic and enkephalinergic pathways project from the PAG to the RVM, and given the existence of multiple serotonin and opioid receptor subtypes localized in the RVM, the aims of this dissertation were to evaluate the effects of intracerebral microinjections of general and specific serotonin and opioid receptor antagonists into the RVM upon morphine antinociception elicited from the PAG.

Microinjections of morphine (2.5 μg) into the PAG produced a significant antinociception on the tail-flick and jump tests. This antinociception was significantly reduced following RVM microinjections of the general serotonergic

antagonist methysergide (0.5-5 μg) on the tail-flick (69%) and jump (50%) tests. To ascertain which serotonergic receptors might be responsible for the above inhibition of morphine's effects, the 5-HT_2 antagonist, ritanserin (0.25-2.5 μg) and the 5-HT_3 antagonist, ICS 205930 (0.25-5 μg) were microinjected into the RVM prior to microinjections of morphine into the PAG. Mesencephalic morphine antinociception was significantly reduced following pretreatment with both ritanserin on the tail-flick (81%) and jump (65%) tests and ICS 205930 on the tail-flick (91%) and jump (63%) tests.

Mesencephalic morphine antinociception was similarly reduced following pretreatment in the RVM with the general opioid receptor antagonist, naltrexone (1-10 μg) on the tail-flick (93%) and jump (89%) tests. Mesencephalic morphine antinociception was also significantly reduced by both the mu-selective antagonist, beta-funaltrexamine (0.5-5 μg) on the tail-flick (93%) and jump (91%) tests, and the $delta_2$-selective antagonist, naltrindole (0.5-5 μg) on the tail-flick (80%) and jump (85%) tests.

In contrast, none of the antagonists were effective in reducing mesencephalic morphine antinociception when they were microinjected into placements lateral or dorsal to the RVM. Further, none of these antagonists produced meaningful changes in basal nociceptive thresholds. These data indicate that ventro-medial medullary serotonergic receptors, specifically the 5-HT_2 and 5-HT_3 receptor subtypes and medullary mu and

delta opioid receptors modulate the transmission of opioid pain-inhibitory signals from the PAG.

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INTRODUCTION

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. Opioid inhibition of nociception occurs at both spinal and supraspinal levels of the neuraxis. This delineation is based upon the antinociceptive properties of morphine or electrical stimulation delivered to specific supraspinal sites (e.g., see reviews: Mayer and Price, 1976; Yaksh and Rudy, 1978a) and the ability of opiates to produce antinociception following intrathecal administration to the spinal cord (Yaksh, 1984a,b).

The study of how supraspinal loci sensitive to opioid antinociception interact with other supraspinal and spinal loci sensitive to opioid antinociception has involved the analysis of neuroanatomical, neurophysiological and neurochemical substrates. Fields and Basbaum (1978) initially described a supraspinal pathway in which opioid antinociception originating in the midbrain periaqueductal gray (PAG) was modulated via an excitatory link to the medullary nucleus raphe magnus (NRM) which in turn projected to the substantia gelatinosa of the spinal cord through the dorsolateral funiculus (DLF). The latter pathway contained serotonin, implicating this transmitter as playing a major

role in pain modulation. Basbaum and Fields (1984) subsequently proposed a role for the nucleus reticularis gigantocellularis (NRGC), the locus coeruleus (LC), and spinally-projecting noradrenergic pathways as also being important in this pain inhibitory system. The PAG sends descending projections to a number of brainstem nuclei which in turn project to the spinal cord, including the LC, NRM, NRGC, nuclei raphe pallidus, reticularis magnocellularis gigantocellularis, and paragigantocellularis lateralis and ventralis (Beitz, Mullett and Weiner, 1983; Mantyh, 1983). Further, reciprocal connections between the LC and the medial medulla exist, providing spinal noradrenergic projections (Moore and Bloom, 1979; Ennis and Aston-Jones, 1987; Clark and Proudfit, 1991; Nygren, Olson and Sieger, 1977).

Electrophysiological studies have further implicated the activation of the rostral ventral medulla (RVM), which includes the NRM and NRGC, in the mediation of opioid antinociception elicited from the PAG (Lovick, West and Wolstencroft, 1978; Pomeroy and Behbehani, 1979; Mohrland and Gebhart, 1980a; Sandkuhler and Gebhart, 1984a; Zorman, Hentall, Adams and Fields, 1981). 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, Barbaro and Heinricher, 1988; Barbaro, Heinricher and Fields, 1989; Fields, Heinricher and Mason, 1991).

Much of our knowledge concerning the neurochemical makeup of pain inhibition has relied largely upon opiate microinjection and electrical stimulation studies, in combination with either lesions or pharmacological manipulations which either increase or decrease the levels of certain neurotransmitter systems. Such studies have implicated a number of different neurotransmitters and peptides in pain modulation, including opioids, serotonin, norepinephrine, acetylcholine, GABA, neurotensin, substance P and the excitatory amino acids. However, little is known about the neurochemical link between the PAG and the RVM.

The aims of this dissertation are to specify which receptors in the RVM serve to mediate opioid antinociception from the PAG. Since the neuroanatomical link between the PAG and the RVM contains serotonin and enkephalins (e.g., Beitz, 1982 a,b), the present studies will determine whether serotonergic or opioid antagonists administered into the RVM will alter the antinociceptive response of morphine administered into the PAG. Since both serotonergic (e.g., see reviews: Peroutka, 1988a,b; Peroutka, Schmidt, Sleight and Harrington, 1990) and opioid (Lord, Waterfield, Hughes and Kosterlitz, 1977; Martin, Eades, Thompson, Huppler and

Gilbert, 1976; Pasternak and Wood, 1986) receptors have specific and distinct subtypes, the present studies will evaluate both general and specific serotonergic and opioid receptor subtypes in these paradigms.

To provide a conceptual basis for the proposed experiments, the following sections provide background information pertaining to: a) opioid peptides and opioid receptor subtypes, including general and specific agonists and antagonists, b) the endogenous opioid antinociceptive system, c) the substrates of spinally- and supraspinally-mediated opioid antinociception, including interactions with monoaminergic systems, and d) a rationale for the present experiments.

A. Opioid Peptides and Opioid Receptor Subtypes:

Opioid Peptides: Opioid peptides are derived from one of three distinct precursor molecules: i) proopiomelanocortin (POMC), ii) proenkephalin, and iii) prodynorphin, all sharing a common opiate-active core (Tyr-Gly-Gly-Phe) (see review: Sherman, Akil and Watson, 1989).

i) POMC: The carboxyl terminus of POMC contains the opioid peptide, beta-endorphin and its precursor beta-lipotropin (beta-LPH). (Eipper and Mains, 1978; Mains, Eipper and Ling, 1977). In the brain, POMC-derived peptides are located in two distinct cell groups (Khachaturian, Lewis, Schafer and Watson, 1985). The first group is in the arcuate and periarcuate nuclei of the hypothalamus (Watson, Akil,

Richard and Barchas, 1978; Bloom, Rossier, Battenberg, Bayon, French, Henricksen, Siggins, Segal, Browne, Ling and Guillemin, 1978). The second group is in the caudal part of the nucleus tractus solitarius (NTS) which projects laterally to the lateral reticular nucleus (Khachaturian et al., 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, NRM, NRGC, NTS, nuclei reticularis lateralis, parabrachialis and ambiguus, and the dorsal motor nucleus of the vagus nerve (Guillemin, Ling and Burgus, 1976; Khachaturian et al., 1985).

ii) Proenkephalin: Within the proenkephalin precursor are several active opioid peptides, including leu-enkephalin, met-enkephalin, met-enkephalin-Arg-Phe and met-enkephalin-Arg-Gly-Leu (Kimura, Lewis, Stern, Rossier, Stein and Udenfriend 1980; Comb, Herbert and Crea, 1982). Enkephalinergic perikarya are found at most levels of the neuraxis, including the telencephalon (cerebral cortex, olfactory tubercle, amygdala, hippocampus, bed nucleus of the stria terminalis and preoptic area), diencephalon (hypothalamus and periventricular and lateral geniculate nuclei of the thalamus), mesencephalon (PAG, superior and inferior colliculi and interpeduncular

nucleus), met-/myelencephalon (NRM, NRGC, NTS, nucleus reticularis paragigantocellularis, lateral reticular nucleus, spinal trigeminal nucleus) 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).

iii) Prodynorphin: 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, Minamino, Chino, Sakakibara and Matsuo, 1981). Several peptide lengths of dynorphin A are biologically active, including dynorphin A_{1-17}, dynorphin A_{1-8} and other peptides of intermediate sizes (Goldstein et al., 1981; Seizinger, Hollt and Herz, 1981; Suda, Tozawa, Tachibana, Demura and Shizume, 1982). Immunoreactive dynorphin perikarya are found in telencephalic (cerebral cortex, striatum, amygdala and hippocampus), diencephalic (suprachiasmatic, paraventricular, supraoptic and arcuate hypothalamus), mesencephalic (PAG), and brainstem areas (spinal trigeminal nucleus, NTS, lateral reticular nucleus), as well as in the dorsal horn of the spinal cord (Khachaturian et al., 1985).

Opioid Receptor Subtypes: Following the discovery of the opiate receptor in 1973 (Pert and Snyder, 1973; Simon, Hiller and Edelman, 1973; Terenius, 1973), Martin and colleagues (1976) suggested that multiple forms of the opiate receptor

existed based on addiction, cross-tolerance and abstinence studies. Three 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. Opioid receptor subtypes are heterogeneously distributed throughout the neuraxis (Goodman, Snyder, Kuhar and Young, 1980; Mansour, Khachaturian, Lewis, Akil and Watson, 1986, 1987). Naltrexone is a long-acting, general opioid receptor antagonist (Zukin and Zukin, 1981).

i) Mu receptors: These 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, Khachaturian, Lewis, Akil and Watson, 1988). In assessing pharmacological effects of the mu receptor,

selective agonists (D-Ala^2, met-Phe^4, Gly (ol) ^{5}-enkephalin, DAMGO: Handa, Lane, Lord, Morgan, Rance and Smith, 1981), and antagonists (beta-funaltrexamine, ß-FNA: Portoghese, Larson, Sayre, Fries, and Takemori, 1980; Takemori, Larson and Portoghese, 1981) have been developed.

ii) Mu_1 receptors: The mu receptor has been further subcharacterized into mu_1 and mu_2 subtypes, based upon pharmacological and biochemical assays (e.g., review by Pasternak and Wood, 1986). The mu_1 binding site is a common high affinity site, binding morphine, ethylketocyclazonine, enkephalin peptides and beta-endorphin with equally high affinity. The mu_2 site selectively binds morphine-like compounds more potently than enkephalins (Wolozin and Pasternak, 1981; Pasternak and Wood, 1986; Clark, Houghten and Pasternak, 1988). Autoradiographic studies demonstrate that mu_1 and mu_2 binding sites have similar, though not identical distributions (Goodman and Pasternak, 1985; Maskowitz and Goodman, 1985a,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.

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 (Mansour et al., 1988). Pharmacological analysis of the delta receptor has utilized the general delta agonists, D-Ser^2,Leu^5-enkephalin-Thr^6 (DSLET) and D-Ala^2,D-Leu^5-enkephalin (DADL) (Lord et al., 1977; Mosberg, Hurst, Hruby, Gee, Yamamura, Galligan and Burks, 1983a) and the general delta antagonist, ICI 174864 (Cotton, Giles, Miller, Shaw and Timms, 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 D-Pen^2, D-Pen^5-enkephalin (DPDPE: Mosberg, Hurst, Hruby, Galligan, Burks, Gee and Yamamura, 1983b) and the long-term antagonist actions of D-Ala^2, Leu^5, Cys^6-enkephalin (DALCE: Bowen, Hellewell, Kelemen, Huey, and Steward, 1987; Jiang, Bowen, Mosberg, Rothman, Porreca, 1990a). The delta$_2$ receptor subtype has been characterized by the agonist actions of D-Ala^2,Glu^4-deltorphan (Jiang, Takemori, Sultana, Portoghese, Bowen, Mosberg and Porreca, 1991) and the antagonist actions of naltrindole (Portoghese, Sultana, Nagase and Takemori, 1988a; Portoghese, Sultana and Takemori, 1988b; Sofuoglu, Portoghese and Takemori, 1991). Indeed, the effects of delta$_1$ and delta$_2$ agonists and antagonists have been dissociated from each other in behavioral assays (Mattia, Vanderah, Mosberg and Porreca, 1991; Jiang et al., 1991).

iv) Kappa receptors: The distribution of kappa opioid receptors include 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), 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). This data indicates 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).

B. Endogenous Opioid Antinociceptive System:

The study of the role of endogenous opioids in the mediation of antinociception utilized three different behavioral techniques: i) microinjection of opiates into discrete brain sites, ii) electrical stimulation of discrete brain areas, and iii) activation of the opioid system following acute exposure to environmental stress.

i. Opiate Microinjection Studies: 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, Yeung and Rudy, 1976, Yaksh and Rudy, 1978a). Moreover, opiate and opioid antinociception in the PAG was reversed by the opiate receptor antagonist naloxone. 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 (Takagi, Satoh, Akaike, Ahibata, and Kuraisi, 1977; 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). A more extensive review of the substrates of these effects follows in the background review of supraspinal antinociception (Section C).

ii) Stimulation-Produced Antinociception (SPA): Following the observation that surgery could be performed in rats during electrical stimulation of the mesencephalic PAG (Reynolds, 1969), the efficacy of SPA has been demonstrated in other brain sites, such as the RVM (Akaike et al., 1978; Besson, Oliveras, Chaouch and Rivot, 1981; Oliveras, Redjemi, Guilbaud and Besson, 1975; Guilbaud, Oliveras, Geisler and Besson, 1977; Satoh, Akaike, Nakazawa and Takagi, 1980). SPA has been

found in rats, cats, rhesus monkeys and man, with analgesic duration increasing as a function of the phylogenetic scale (Mayer and Price, 1976; Mayer and Liebeskind, 1974; Oliveras, Besson, Guilbauld and Liebeskind, 1974; Richardson and Akil, 1977). Mapping studies showed that central morphine antinociception and SPA had a close correspondence (Jacquet and Lajtha, 1974; Sharpe, Garnet and Cicero, 1974; Mayer and Price, 1976). Similar mechanisms of action for morphine antinociception and SPA have been suggested. Repeated stimulation of the PAG produces tolerance to the antinociceptive response, and morphine-tolerant rats displayed increases in the threshold for PAG SPA (Mayer and Hayes, 1975). Ineffective levels of PAG stimulation paired with subanalgesic doses of morphine produces an interactive antinociception (Samamin and Valzelli, 1971). Further support for the idea that SPA and central morphine antinociception activated a common endogenous pain-inhibitory system included the observations that lesions placed in the dorsolateral funiculus of the spinal cord blocked both SPA and morphine antinociception (Basbaum, Morley, O'Keefe and Clanton, 1977) and that both forms of antinociception elicited from the PAG produced similar excitatory actions upon NRM neurons (Oleson, Twombly and Liebeskind, 1978). Subsequent studies found that the substrates mediating PAG SPA were dependent upon the locus of stimulation. Whereas SPA elicited from ventral PAG sites was blocked by either naloxone or NRM lesions, SPA elicited

from dorsal PAG sites was unaffected by these manipulations (Cannon, Prieto, Lee and Liebeskind, 1982; Prieto, Cannon and Liebeskind, 1983; Thorn, Applegate and Johnson, 1989). Also, tolerance to the antinociceptive effect of ventral stimulation is obtained more rapidly than that of dorsal stimulation (Morgan and Liebeskind, 1987). These results complement the suggestion that different anatomical pathways for SPA existed in neurophysiological studies (Lovick, 1985).

iii) Stress-Induced Antinociception (SIA): The functional significance of endogenous opioid and nonopioid antinociceptive systems was confirmed by the findings that acute exposure to a number of stressors (e.g., rotation, inescapable footshock, cold-water swims, glucoprivation, restraint and hypertonic saline injections) produced a transient antinociceptive response (Amir and Amit, 1978; Bodnar, Kelly, Steiner and Glusman, 1978a; Bodnar, Kelly, Brutus, Mansour and Glusman, 1978b; Chance, White, Krynock, and Rosecrans, 1977; Hayes, Bennett, Newlon and Mayer, 1978; Madden, Akil, Patrick and Barchas, 1977). Like morphine tolerance, repeated exposure to a number of stressors (e.g., cold-water swims, inescapable footshock, glucoprivation) produced adaptation to the antinociceptive response (Bodnar, Kelly, Spiaggia and Glusman, 1978c; Bodnar, Kelly, Brutus and Glusman, 1978d; Chance and Rosecrans, 1979). However, whereas the antinociception elicited by some stressors (inescapable footshock) was cross-tolerant with morphine antinociception

and significantly reduced by naloxone (Chesher and Chan, 1977; Hayes et al., 1978), the antinociception elicited by other stressors (cold-water swims, conditioning) was impervious to these manipulations (Bodnar et al., 1978a; Bodnar, Kelly, Spiaggia, Ehrenberg and Glusman, 1978e). Exposure to different parameters of the same stressor was found to selectively activate opioid or nonopioid pain-inhibitory systems. Antinociception following prolonged intermittent footshock was reduced by naloxone pretreatment and morphine tolerance, whereas antinociception following brief continuous footshock was impervious to these manipulations (Lewis, Cannon and Liebeskind, 1980; Lewis, Sherman and Liebeskind, 1981). Similarly, whereas continuous cold-water swim antinociception was unaffected by morphine tolerance and naloxone pretreatment (Bodnar et al., 1978a,e), intermittent cold-water swim antinociception was significantly reduced by both of these manipulations (Girardot and Holloway, 1984a,b). Thus, the type of stressful situation and the parameters of the stressful situation dictate the activation of opioid and nonopioid systems (Hayes et al., 1978; Hayes and Katayama, 1986; Lewis et al., 1980, 1981; Bodnar et al., 1978a,e; Bodnar and Sikorszky, 1983; Watkins and Mayer, 1982; Grau, Hyson, Maier, Madden and Barchas, 1981; Girardot and Hollaway, 1984 a,b) and their interaction with opioid forms of PAG SPA (Terman, Penner and Liebeskind, 1985). Further studies, including manipulations of the hypothalamic-hypophyseal-

adrenal axis, lesions of the PAG and NRM, and the use of selective transmitter antagonists and agonists have resulted in differential characterization of SIA as opioid-neurohormonal, opioid-neural, nonopioid-neural, and nonopioid-neurohormonal (e.g., Watkins and Mayer, 1982; Bodnar, Kelly, Brutus and Glusman, 1979; Bodnar, 1990).

C. Spinal and Supraspinal Opioid Antinociception:

Spinal System: Intrathecal administration of opiates and opioid analogues produces a dose-dependent antinociception which is blocked by naloxone (Yaksh and Rudy, 1978b; 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. These studies have found that 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, Bodnar, Gistrak and Pasternak, 1989). Delta and kappa agonists produce predominantly spinal antinociception which is blocked by selective antagonists for these receptors (Yaksh, 1984a,b; Porreca, Mosberg, Hurst, Hruby and Burks, 1984; Porreca, Heyman, Mosberg, Omnaas and Vaught, 1987; Wuster, Schulz and Herz, 1980; Heyman, Mulvaney, Mosberg and Porreca, 1987, Heyman, Vaught, Raffa and Porreca, 1988; Jiang, Heyman, Sheldon, Portoghese, Bowen, Mosberg and

Porreca, 1990b). 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). Studies which have focused 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 have 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 observed by 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, 1978b; Vasko, Pang and Vogt, 1984). Thus, further work should be performed to delineate the role of serotonin and morphine.

Supraspinal System: 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, and project to the substantia gelatinosa of the spinal cord through the dorsolateral funiculus (Basbaum and Fields, 1984; Fields and Basbaum, 1978). This model of descending pain inhibition is supported by various lines of research. Lesions placed in either the NRM (Proudfit and Anderson, 1975; Mohrland and Gebhart, 1980b) or the dorsolateral funiculus (Kitahata, Yosaka, Bonikos and Hoffert, 1974; Le Bars, Menetrey, Conseiller and Besson, 1975; Murphin, Bennett and Mayer, 1976) attenuate the 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 et al., 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 supraspinal antinociceptive system, ii.) a physiological analysis of supraspinal antinociception, and iii.) a pharmacological analysis of the supraspinal antinociceptive system, including the opioid and monoaminergic systems.

<u>i) Anatomical Substrates of Supraspinal Antinociception: Periaqueductal Gray (PAG):</u> The midbrain PAG area surrounding the cerebral aqueduct is a functionally heterogenous region with respect to both its cytoarchitecture and its connectivity with other brain regions (Altman and Bayer, 1981;

Hamilton, 1973; Beitz, 1985). Beitz (1985) has characterized the PAG as consisting of four distinct anatomical subdivisions referred to as the medial, dorsolateral, ventrolateral and dorsal divisions. As mentioned, antinociception appears to be most effective following electrical stimulation or opiate microinjection of the ventrolateral region of the PAG (Yaksh et al., 1976; Lewis and Gebhart, 1977; Akil, Watson, Young, Lewis, Khachaturian and Walker, 1984). Afferent projections to the PAG are extensive and include cortical (prefrontal cortex, basal forebrain), diencephalic (lateral and ventromedial hypothalamic nuclei, lateral habenula nucleus), and mesencephalic (nucleus cuneiformis and substantia nigra) input (Beitz, 1982c, Mantyh, 1983). The PAG also receives predominant input from limbic areas (amygdala) and many brainstem sites (pontine reticular formation, nucleus solitarus, LC, NRM, NRGC, NRPG) (Beitz, 1982c).

Though retrograde tracer studies have demonstrated direct PAG-spinal projections (Castiglioni, Gallaway and Coulter, 1978; Mantyh and Peschanski, 1982), the PAG and adjacent nucleus cuneiformis projections to medullary structures (NRM, NRGC, raphe paragigantocellularis lateralis and ventralis) (Abols and Basbaum, 1981; Beitz et al., 1983; Mantyh, 1983; Van Bockstaele, Pieribone and Aston-Jones, 1989; Van Bockstaele, Aston-Jones, Pieribone, Ennis and Shipley, 1991) serve as important relays for midbrain modulatory influences upon spinal nociceptive transmission (Fields and Basbaum,

1978; Basbaum and Fields, 1984). Both enkephalinergic-immunoreactive and serotonergic neurons project from the PAG to the RVM (Beitz, 1982a,b; Lakos and Basbaum, 1988).

Rostral Ventral Medulla (RVM): The RVM consists of the midline NRM, which extends from the rostral pole of the inferior olive to the rostral pole of the superior olive, the adjacent nucleus reticularis magnocellularis, the lateral reticular nucleus, and the nucleus reticularis paragigantocellularis, which is located ventral and lateral to the reticularis magnocellularis. The most ventromedial portion of the paragigantocellularis corresponds to the nucleus reticularis gigantocellularis, pars alpha (Abols and Basbaum, 1981). Enkephalinergic cells serve as intrinsic interneurons in the RVM (Lewis, Khachaturian and Watson, 1985). The nuclei of the RVM receive input from other areas within the medullary and pontine reticular formation, including noradrenergic projections from the LC (Gallager and Pert, 1978; Moore and Bloom, 1979). Additionally, the nucleus paragigantocellularis is a major afferent to the LC (Aston-Jones, Ennis, Pieribone, Nickell and Shipley, 1986; Guyenet and Young, 1987; Pieribone and Aston-Jones, 1988). The hypothalamus, frontal cortex, amygdala and the bed nucleus of the stria terminalis also project to the RVM (Holstege, 1987). Moreover, a prominent input to the RVM derives from the PAG and the nucleus cuneiformis (Ruda, 1975; Abols and Basbaum, 1981; Edwards, 1975; Chung, Kevetter, Yezierski, Haber, Martin

and Willis, 1983; Van Bockstaele et al., 1989, 1991; Beitz et al., 1983). The PAG projections are mainly from the dorsal and ventrolateral subdivisions (Abols and Basbaum, 1981; Chung et al., 1983; Beitz, 1985; Van Bockstaele et al., 1991). Thus, the PAG-RVM pathway provides anatomical evidence for the idea that the antinociception elicited from the microinjection of opiates or stimulation of the PAG involves a connection between the PAG and the RVM. The RVM in turn projects to the solitary nucleus, the dorsal motor nucleus and the marginal and gelatinous layers of the trigeminal nucleus caudalis and via the spinal dorsolateral funiculus to the spinal dorsal horn (Abols and Basbaum, 1981; Holstege, 1987), terminating in laminae I, II, V and VII, which contain the terminals of small diameter nociceptive primary afferents (Fields and Basbaum, 1978).

ii) Physiological Circuitry Involved in Supraspinal Antinociception: Three physiologically-distinct classes of neurons can be identified in the RVM based upon the temporal correlation of changes in their firing with the execution of reflexes elicited by noxious stimulation (Fields et al., 1988). Cells of the first class, "on-cells," reliably show a sudden increase in firing just prior to the occurrence of a response, which are activated to evoke a withdrawal reflex (Vanegas et al., 1984). "On-cells" are highly active just prior to and during the execution of the tail-flick test (D'Amour and Smith, 1941), which is one of the nociceptive

measures 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 suppresses "on-cell" firing (Barbaro et al., 1986; Cheng et al., 1986).

The second set of cells, "off-cells," show 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). 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., 1984).

"On" and "off" cells play a central role in descending nociceptive modulation, and both cell classes are excited by electrical stimulation in the PAG (Vanegas et al., 1984). Thus, any observed enhancement of nociception that is correlated with an increase in "on-cell" activity could be explained by the removal of descending inhibition exerted by "off cells." 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 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 investigators (Fields et al., 1988; Vanegas et al., 1984; Mason and Fields, 1989) believe modulation of nociception by the RVM must be interpreted in terms of the interactions between these two populations of RVM cells and their termination in the dorsal horn.

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 and project to the spinal cord, the vast majority of cells are classified as either "on" or "off-cells." In the basic model of RVM circuitry, Fields et al. (1988) favor the view that "off-cells" excite other "off-cells" and inhibit "on-cells" throughout the RVM, thereby resulting in antinociception.

iii) Pharmacology of Supraspinal Antinociception: 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, LC,

NRM and NRGC elicits antinociception which can be blocked by naloxonazine (Bodnar, Williams, Lee and Pasternak, 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, LC, NRM or NRGC (Bodnar et al., 1988). Kappa receptors do not appear participate in supraspinal antinociception. Supraspinal administration of selective kappa receptor ligands fails to elicit antinociception (Friedman, Jen, Chang, Lee and Loh, 1981; Chavkin, James and Goldstein, 1982; Fang, Haws, Drasner, Williamson and Fields, 1989). Ethylketcyclazocine (EKC), one of the original prototypical kappa agonists produces antinociception following intraventricular administration, but not following administration into either the PAG or LC. Indeed, EKC pretreatment into these structures interferes with the subsequent development of morphine antinociception. EKC's actions upon morphine antinociception is that of a partial mu_1 agonist because intraventricular EKC antinociception is blocked by naloxonazine, and because simultaneous administration of EKC into the PAG and LC produces a naloxonazine-sensitive antinociception (Bodnar, Paul and Pasternak, 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 LC (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 LC 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 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. Tennen (1968) initially recognized the importance of serotonin in the mediation of opiate antinociception when he found that reducing serotonin levels

with para-chlorophenylalanin attenuated the antinociceptive effect of morphine. This effect could be reversed with the 5-HT precursor, 5-hydroxytryptophan (5-HTP). Further, it has been shown that the antinociception produced by microinjection of morphine into the PAG results in the release of 5-HT from the spinal cord (Yaksh and Tyce, 1979). The above mentioned studies using serotonergic antagonists further demonstrated the involvement of descending serotonergic pathways in the mediation of supraspinal opioid antinociception. The recent discoveries of the existence of multiple serotonin receptors, categorized into 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C}, 5-HT_{1D}, 5-HT_2, and 5-HT_3 subtypes (Bradley, Engel, Fenuik, Fozard, Humphrey, Middlemiss, Mylecharane, Richardson and Saxena, 1986; Glennon, 1987; Peroutka, 1988a,b; Peroutka et al., 1990) has raised the question as to which subtype(s) participate in the mediation of supraspinal opioid antinociception.

5-HT_1 Receptors: All of the specific subtypes of the 5-HT_1 receptor are labelled by [^{3}H] 5-HT. The specific subtypes of the 5HT_1 receptor, 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C} and 5-HT_{1D} are labelled with [^{3}H]-OH-DPAT (Gozlan, El Mestikawy, Pichat, Glowinski and Hamon, 1983), [125I] iodocyanopindolol (Hoyer, Engel and Kalkman, 1985), [^{3}H] mesulergine (Pazos, Hoyer and Palacios, 1984), and [^{3}H] 5-HT (Heuring and Peroutka, 1987) respectively, although other ligands have been used in studies of receptor binding.

In terms of their distribution, the hippocampus (CA1

region and dentate gyrus), septum, amygdaloid regions and the raphe (particularly dorsal and median raphe) nuclei show dense 5-HT_{1A} binding. This receptor subtype has also been found in the neocortex, ventral tegmental area and hypothalamus (Hoyer, Pazos, Probst and Palacios, 1986; Verge, Davel, Marcinkiewicz, Patey, El Mestikawy, Gozlan and Hamon, 1986; Pazos, Probst and Palacios, 1987). Also, 5-HT_{1A} receptors have been localized in the dorsal horns of the spinal cord (Hamon, Lanfumey, El Mestikawy, Boni, Miquel, Bolanos, Schechter and Gozlan, 1990). These latter investigators believe that the presence of these receptors in the spinal cord may indicate that this receptor subtype plays a role in nociception.

The 5-HT_{1B} receptor has not been found in the guinea pig, pig, calf, cat, frog, monkey, human or pigeon brain (Peroutka et al., 1990). In the rodent brain, the highest density of 5-HT_{1B} receptors has been found in substantia nigra, globus pallidus, dorsal subiculum and superior colliculi (Pazos and Palacios, 1985). The 5-HT_{1C} receptor shares pharmacological characteristics with the 5-HT_2 receptor (Hartig, Hoffman, Kaufman and Hirata, 1990), leading some investigators to propose that 5-HT_{1C} receptors might be related to 5-HT_2 receptors (Hoyer, Waeber, Pazos, Probst and Palacios, 1988). 5-HT_{1C} receptors are densely distributed in the choroid plexus, and are present at low levels in the globus pallidus, substantia nigra, cerebral cortex and olfactory turbercules in rodents, pigs and humans (Yagaloff and Hartig, 1985; Pazos et

al., 1987). Also, 5-HT_{1C} receptors have been found in the spinal cord (Huang and Peroutka, 1987; Daval, Verge, Basbaum, Bourgoin and Hamon, 1987; Zemlan, Schwab, Murphy and Behbehani, 1990).

The 5-HT_{1D} receptor does not exist in the rodent brain. In mammals (e.g., guinea pig, pig, pigeon, rabbit, dog, calf, monkey and human) these receptors are most dense in the basal ganglia and substantia nigra, accounting for 90% of the 5-HT_1 binding (Waeber and Palacios, 1990). A lower density of 5-HT_{1D} receptors occurs in the hippocampus, cortex and raphe (Waeber, Zhang and Palacios, 1990).

5-HT_2 Receptors: The most commonly used radioligand for 5-HT_2 receptors is [^{3}H] ketanserin (Leysen, Niemegeers, Van Nueten and Laduron, 1982). The anatomical distribution of 5-HT_2 receptors is somewhat similar in both the human and rodent brain (Peroutka et al., 1990) and is most dense in laminae I and V of the neocortex, olfactory tubercle, hypothalamus, layers CA1 and CA3 of the hippocampus, parts of the basal ganglia (caudate, putamen and nucleus accumbens) and the dorsal raphe nucleus (Slater and Patel, 1983; Pazos, Cortés and Palacios, 1985). 5-HT_2 receptors appear at low densities in the midbrain and cerebellum, and are not found in the spinal cord (Leysen et al., 1982; Monroe and Smith, 1983; Zemlan et al., 1990).

5-HT_3 Receptors: This receptor subtype has been labelled using [^{3}H] GR 65630 and [^{3}H] ICS 205930 (Kilpatrick, Jones and

Tyers, 1987; Hoyer and Neijt, 1987). 5-HT_3 receptors are densely distributed in the nucleus tractus solitarius, dorsal nucleus of the vagus nerve, spinal trigeminal nucleus, area postrema, as well as in the substantia gelatinosa of the spinal cord. Moderate densities appear in the hippocampus and the amygdala (Hoyer, Waeber, Karpf, Neijt and Palacios, 1989; Waeber, Dixon, Hoyer and Palacios, 1988; Glaum and Anderson, 1988; Kilpatrick, Jones and Tyers, 1988).

Role of 5-HT Receptor Subtypes in Opioid Antinociception: The participation of 5-HT receptor subtypes in the mediation of opioid antinociception has only recently been investigated with the use of selective agonists and antagonists for the specific subtypes. This research has shown that systemic administration of the 5-HT_{1A} agonists, buspirone, ipsapirone and gepirone attenuates systemic opiate antinociception, while agonists at the 5-HT_{1B} and 5-HT_{1C} and antagonists at the 5-HT_{1A} site do not affect opiate antinociception (Millan and Colpaert, 1990). 5-HT_2 receptors have been implicated in the mediation of opiate and opioid-mediated antinociception in systemic studies (Kiefel, Paul and Bodnar, 1989; Paul, Manna, Pfaus and Pinel, 1988; Paul and Phillips, 1986; Paul and Pinel, 1990).

D. Rationale:

The present series of studies have resulted in published work. The first study examined whether the general 5-HT antagonist, methysergide, administered into the RVM would

alter morphine antinociception elicited from the PAG as measured by the tail-flick and jump tests (Kiefel, Cooper and Bodnar, 1991). The second study examined whether either the specific 5-HT_2 antagonist, ritanserin or the specific 5-HT_3 antagonist, ICS 205930 administered into the RVM would alter morphine antinociception elicited from the PAG (Kiefel, Cooper and Bodnar, 1992). The third study examined whether either the general opioid antagonist, naltrexone, the mu-selective antagonist, beta-funaltrexamine, or the $delta_2$-selective antagonist, naltrindole administered into the RVM would alter morphine antinociception elicited from the PAG (Kiefel, Rossi and Bodnar, 1993). Background information relevant to these specific experiments follows.

1. RVM Methysergide/PAG Morphine Antinociception: The proposed neural substrates of supraspinal opioid antinociception originates in the midbrain PAG, synapses in the medullary NRM, and projects to the substantia gelatinosa of the spinal cord through the dorsolateral funiculus (Fields and Basbaum, 1989; Basbaum and Fields, 1984). The NRGC and the NRGC, pars alpha (Aimone and Gebhart, 1986; Azami et al., 1982; Sandkuhler and Gebhart, 1984a; Satoh, Kubota, Iwama, Wada, Yasui, Fujibayashi and Takagi, 1983) have also been implicated in the medullary mediation of supraspinal opioid antinociception. Electrical stimulation of these medullary loci produces antinociception (Sandkuhler and Gebhart, 1984b; Zorman et al., 1981), and their physiological firing

characteristics predicted supraspinal antinociception following electrical stimulation and morphine (e.g., Fields et al., 1983, 1988, 1991; Vanegas et al., 1984; Cheng et al., 1986; Barbaro et al., 1986, 1989; Zhuo and Gebhart, 1990). Spinal serotonergic synapses partially modulate supraspinal opioid antinociception (Jensen and Yaksh, 1986b; Proudfit and Hammond, 1981; Tseng and Tang, 1989; Yaksh, 1979) as well as the antinociception elicited by electrical stimulation of medullary and PAG loci (Barbaro et al., 1989; Hammond and Yaksh, 1984).

The neurochemical makeup of the PAG-RVM pathway activated by mesencephalic opioid receptors has not been clearly defined. Direct PAG-NRGC projections (Beitz et al., 1983; Van Bockstaele et al., 1989) and direct PAG-NRM projections (Abols and Basbaum, 1981; Beitz et al., 1983; Gallerger and Pert, 1978) have been described with 55-63% of the confirmed neurons in the latter pathway containing serotonin (Beitz, 1982a). Autoradiography has confirmed serotonin receptors on medial medullary neurons (Pazos and Palacios, 1985; Pazos et al., 1985). Injections of serotonin or serotonergic agonists into the NRM increases nociceptive thresholds (Llewelyn, Azami and Roberts, 1983) and methysergide administered into the NRM blocks the antinociception induced by electrical stimulation of the PAG (Aimone and Gebhart, 1986). To assess whether a serotonergic medullary synapse mediates morphine antinociception elicited from the PAG, the present study

determined whether microinjections of methysergide into the RVM would alter the antinociception elicited by morphine microinjections into the PAG, as measured by the tail-flick (D'Amour and Smith, 1941) and jump (Evans, 1961) tests.

2. RVM Ritanserin and ICS 205930/PAG Morphine Antinociception: The first study (Kiefel et al., 1991) investigated whether a serotonergic medullary synapse mediates morphine antinociception elicited from the PAG by evaluating the effects of medullary methysergide upon mesencephalic morphine antinociception on the tail-flick and jump tests in rats. Multiple serotonin receptors have been classified into $5\text{-}HT_{1A}$, $5\text{-}HT_{1B}$, $5\text{-}HT_{1C}$, $5\text{-}HT_{1D}$, $5\text{-}HT_2$ and $5\text{-}HT_3$ subtypes (e.g., see reviews: Peroutka 1988a,b; Peroutka et al., 1990). 5-HT receptor subtype pharmacology is quite "fluid," (i.e. subject to change) and that specificity of the drugs for a subtype is subject to further knowledge. We can only say with certainty that a drug exerts an effect, but we can only interpret the effect based on the available data regarding the receptor subtype which it is working. Autoradiography has confirmed the existence of both $5\text{-}HT_2$ and $5\text{-}HT_3$ receptors on medial medullary neurons (Pazos et al., 1985; Waeber et al., 1988). Also, both the $5\text{-}HT_2$ and the $5\text{-}HT_3$ receptor subtypes have been implicated in the mediation of opioid antinociception (Kiefel et al., 1989; Paul et al., 1986; 1988; 1990; Ho and Takemori, 1990). To assess whether medullary $5\text{-}HT_2$ and $5\text{-}HT_3$ receptor subtypes mediate morphine antinociception elicited from the

PAG, the present study determined whether microinjections of either the 5-HT_2 receptor antagonist, ritanserin or the 5-HT_3 receptor antagonist, ICS 205930 into the RVM would alter the antinociception elicited by morphine microinjections into the PAG, as measured by the tail-flick and jump tests.

3. RVM Naltrexone, Beta-Funaltrexamine and Naltrindole/PAG Morphine Antinociception: The first two studies (Kiefel et al., 1991, 1992) examined participation of serotonergic medullary synapses in the mediation of morphine antinociception elicited from the PAG by pretreatment in the RVM with either the general 5-HT antagonist, methysergide, the selective 5-HT_2 antagonist, ritanserin, or the selective 5-HT_3 antagonist, ICS 205930. These studies evaluated the effects of the antagonists to alter nociceptive latencies or thresholds themselves, and whether medullary cannula placements that were either lateral, dorsal or ventral to the RVM elicited antagonistic effects upon mesencephalic morphine antinociception.

As mentioned, enkephalinergic-immunoreactive neurons project from the PAG to the RVM (Beitz, 1982b), and also serve as intrinsic interneurons in the RVM (Lewis et al., 1985). Opiate receptors have been localized in each of these nuclei as well (Atweh and Kuhar, 1977a,b; Goodman et al., 1980; Lewis et al., 1985). Antinociceptive responses are elicited from the RVM following morphine, mu-selective agonists and delta-selective agonists (Azami et al., 1982; Bodnar et al., 1988;

Satoh et al., 1983; Zorman et al., 1981) which is consistent with the observations that both mu and delta receptors are present in these structures (Goodman et al., 1980; Moskowitz and Goodman, 1985a,b). In addition to the short-acting and general opioid antagonist, naltrexone (Zukin and Zukin, 1981), the mu-selective antagonist, beta-funaltrexamine (ß-FNA: Portoghese et al., 1980) and the $delta_2$-selective antagonist, naltrindole (Portoghese et al., 1988) have been shown to be effective in antinociceptive assays (Jiang et al., 1990b; Paul et al., 1989; Sofuoglu et al., 1991; Takemori et al., 1981; Ward, Portoghese and Takemori, 1982; Ward and Takemori, 1983). To ascertain whether an opioid synapse in the RVM participates in the mediation of morphine antinociception elicited from the PAG, this study examined whether mesencephalic morphine antinociception on the tail-flick and jump tests was altered following pretreatment in the RVM of either naltrexone, ß-FNA or naltrindole. Further, to ascertain the anatomical specificity of such effects, peak doses of each antagonist were administered into sites lateral and dorsal to the RVM to determine their actions upon mesencephalic morphine antinociception. As mentioned previously, the specificity of these compounds for the mu and delta receptor is subject to change.

GENERAL METHODS

Subjects: Adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, 80-120 days of age) weighing between 300-600 grams served as subjects in all experiments. Animals were housed individually 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 schedule at ambient room temperatures between 22 and 25°C.

Surgery: Before surgery, rats were pretreated with chlorpromazine HCL (3 mg/kg, i.p.) 20 min prior to anesthesia with ketamine HCL (100 mg/kg, i.m.) and stereotaxically implanted (Kopf Instruments) with two stainless steel guide cannulae (26 gauge, Plastic Products Co., Roanoke VA). One cannula was aimed at the PAG and the second one was aimed at the RVM (NRM/NRGC). The stereotaxic coordinates were taken from Paxinos and Watson (1982) and were modified as proven necessary. With the incisor bar set at -5 mm, the coordinates for the PAG were: 0.3-0.6 mm anterior to the lambda suture, 1.5-2.0 mm lateral to the sagittal suture, 6.5-6.8 mm from the top of the skull, and angled at 12° toward the sagittal plane. Coordinates for the NRM were: 10.8-11.3 mm posterior to the bregma suture, at the midline, and 9.5-11.0 mm from the top of the skull. The NRGC coordinates were: 10.8 mm posterior to bregma, 0.7 mm lateral to the midline, and 9.5 mm from the top of the skull. Additional groups of control rats were

stereotaxically implanted with cannulae aimed at the PAG and medullary placements that were lateral (1.5-2.0 mm lateral to the midline) and dorsal (9.5-10.0 mm from the top of the skull) to the RVM. The cannulae were secured to three stainless steel anchor screws with dental acrylic, and kept patent between paradigms with 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: Morphine Hcl (2.5 μg, Pennick Laboratories) was infused intracerebrally into the PAG of all animals in all of the experiments. The morphine was dissolved in normal 0.9% saline. This morphine dose was chosen because of its significant, but not maximal antinociceptive effects following PAG microinjection (e.g., Bodnar et al., 1988).

In the serotonin antagonist studies (Experiments 1 and 2), the general 5-HT antagonist, methysergide (0.5-5 μg, Sandoz Laboratory, East Hanover, New Jersey), the 5-HT_2 antagonist, ritanserin (0.25-2.5 μg, Janssen, Beerse, Belgium), and the 5-HT_3 antagonist, ICS 205930 (0.25-5 μg, Sandoz Laboratory, Basle, Switzerland) were infused intracerebrally into the RVM. The methysergide was dissolved in 0.9% normal saline. The ritanserin was initially prepared in 100% methanol at a concentration of 10 μg/μl and then titrated with 0.9% normal saline to its desired concentration 0.5 h prior to treatment. ICS 205930 was initially prepared

in 100% dimethyl sulfoxide (DMSO) at a concentration of 10 μg/μl and then titrated with 0.9% normal saline to its desired concentration 0.5 h prior to treatment. In the opioid antagonist study (Experiment 3), the general opioid antagonist, naltrexone (1.0-10 μg, Sigma Chemical Company, St. Louis, MO), the specific mu antagonist, ß-FNA (0.5 and 5.0 μg, Research Biochemicals, Natick, MA), and the specific $delta_2$ antagonist, naltrindole (0.5 and 5.0 μg, Research Biochemicals) were infused intracerebrally into the RVM. Naltrexone and ß-FNA were dissolved in normal saline. Naltrindole was dissolved in 45% (w/v) aqueous 2-hydroxpropyl-ß-cyclodextrin buffer in distilled water.

Vehicle injections in all experiments consisted of normal saline (1 μl). All microinfusions were administered in 1 μl volumes injected continuously through a stainless steel internal cannula which was connected to a Hamilton microsyringe by polyethylene tubing.

Nociceptive Tests: All animals were tested on the tail-flick test (D'Amour and Smith, 1941) which was immediately followed by the jump test (Evans, 1961). This order was maintained so that carry-over effects between the two tests would be minimized. Baseline measures on both of these tests were determined over at least four days prior to the initiation of any experimental conditions in order to establish stability of nociceptive thresholds. All animals displayed consistent latencies and thresholds in baseline and

vehicle testing, and did not appear subject to desensitization.

1. Tail-flick Test: This test (D'Amour and Smith, 1941) measures reactivity to thermal heat directed at the tail. The stimulus source (IITC Company, Woodland, CA.) was mounted 8 cm dorsal and 4-10 cm proximal to the tip of the tail of a lightly restrained animal. The onset of the radiant heat stimulus activates a digital timer which is stopped by the withdrawal of the animal's tail. Tail-flick latency is described as the time elapsing between the onset of the radiant heat stimulus and the withdrawal response of the animals. The intensity of the thermal stimulus was set to produce stable baseline tail-flick latencies of between 2.5 and 3.5 sec. Each tail-flick session consisted of four latency determinations made at 10-sec intervals. In order to avoid tissue damage, a trial was automatically terminated if the animal did not respond within 15 sec. The mean of the last three of the four determinations in sec constituted the final latency score for that trial.

2. Jump Test: This measure of reactivity to electric shock (Evans, 1961) was assessed by placing each unrestrained animal in a 30 cm by 24 cm plexiglass chamber with a floor consisting of 16 grids 1.5 cm apart. Electric shock was delivered to the grids by a shock generator (BRS/LVE) through a shock scrambler (Campden Instruments). Using an ascending method of limits procedure, the jump threshold was defined in

Ma as the lowest of two consecutive intensities in which the animal simultaneously removed both hindpaws from the grids. Each trial began with the animal receiving a 300 msec footshock at a current intensity of 0.10 Ma. Subsequent shocks were increased in 0.05 Ma increments at 10-sec. intervals until the jump threshold was determined. After each trial, the current intensity was reset to 0.10 Ma and the procedure was repeated until six trials were completed, with the threshold value determined as the mean intensity of these six trials.

Statistical Analysis: Split-plot analysis of variance assessed significant effects among conditions and across each experimental time course with Dunnett tests ($p<.05$) comparing morphine antinociception relative to vehicle/vehicle treatment. The analysis of variance was separate for the tail-flick and jump tests. Dunn comparisons ($p<.05$) identified antagonist effects relative to vehicle/morphine treatment. The peak antinociceptive effect for each nociceptive test in each protocol was derived by subtracting the vehicle/vehicle score at 30 min from each respective experimental score at 30 min. The total antinociceptive effect for each nociceptive test in each protocol was derived by subtracting the sum of the four vehicle/vehicle scores (30-120 min) from the sum of each of the respective experimental scores (30-120 min). Alterations in either peak or total morphine antinociception as a function of antagonist

treatments are expressed as a percentage utilizing the formula (1 -antagonist-morphine score ÷ vehicle-morphine score). If a drug still exerts significant effects at low doses, one might not be able to tell an ID_{50}.

Histological Procedures: After testing, cannulae placements were examined in anesthetized (Euthanasia, H. Schein) rats who then received a transcardic perfusion with 0.9% normal saline followed by 10% buffered formalin. The brains were then removed and sliced in 40 μm sections through the cannulae placements. The sections were subsequently stained with Cresyl violet and examined by light microscopy by an observer unfamiliar with the behavioral data. Only animals with confirmed cannulae placements were included in the data analysis.

EXPERIMENT 1: RVM METHYSERGIDE/PAG MORPHINE ANTINOCICEPTION

The proposed neural substrates of supraspinal opioid antinociception originates in the midbrain PAG, synapses in the medullary NRM, and projects to the substantia gelatinosa of the spinal cord through the dorsolateral funiculus (Fields and Basbaum, 1989; Basbaum and Fields, 1984). The NRGC and the NRGC, pars alpha (Aimone and Gebhart, 1986; Azami et al., 1982; Sandkuhler and Gebhart, 1984a; Satoh et al., 1983) have also been implicated in the medullary mediation of supraspinal opioid antinociception. The neurochemical makeup of the PAG-RVM pathway activated by mesencephalic opioid receptors has not been clearly defined. Direct PAG-NRGC projections (Beitz et al., 1983; Van Bockstaele et al., 1989) and direct PAG-NRM projections (Abols and Basbaum, 1981; Beitz et al., 1983; Gallerger and Pert, 1978) have been described with 55-63% of the confirmed neurons in the latter pathway containing serotonin (Beitz, 1982a). Autoradiography has confirmed serotonin receptors on medial medullary neurons (Pazos and Palacios, 1985; Pazos et al., 1985). To assess whether a serotonergic medullary synapse mediates morphine antinociception elicited from the PAG, the present study determined whether microinjections of methysergide into the RVM would alter the antinociception elicited by morphine microinjections into the PAG, as measured by the tail-flick and jump tests.

Protocol

Each rat received pairs of microinjection conditions at weekly intervals. Tail-flick latencies and jump thresholds were determined at 30, 60, 90 and 120 min following the second microinjection of each pair. Table 1 summarizes the experimental treatments, doses and sample sizes used to assess the effects of medullary methysergide upon morphine antinociception elicited from the PAG. Medullary microinjections preceded mesencephalic microinjections by 15 min to allow methysergide effects to develop (Kiefel et al., 1989). A weekly interval separated conditions in order to minimize possible tolerance effects (e.g., Bodnar et al., 1988). Multiple injections were necessary to determine antagonist effects as a function of precise PAG and RVM placements.

Results

Figure 1 illustrates cannula placements in the PAG (upper panel) and the medulla (lower panel). Mesencephalic cannula placements were all localized in the lateral, ventral and ventrolateral quadrants of the PAG and immediately-adjacent tegmentum as far rostral as the III cranial nerve nucleus and as far caudal as the dorsal raphe nucleus (Paxinos and Watson, 1986), and were each capable of eliciting morphine antinociception. In 14 rats, medullary cannula placements were localized in either the NRM, NRGC or NRGC, pars alpha as far rostral as the genu of the VII cranial nerve and as far

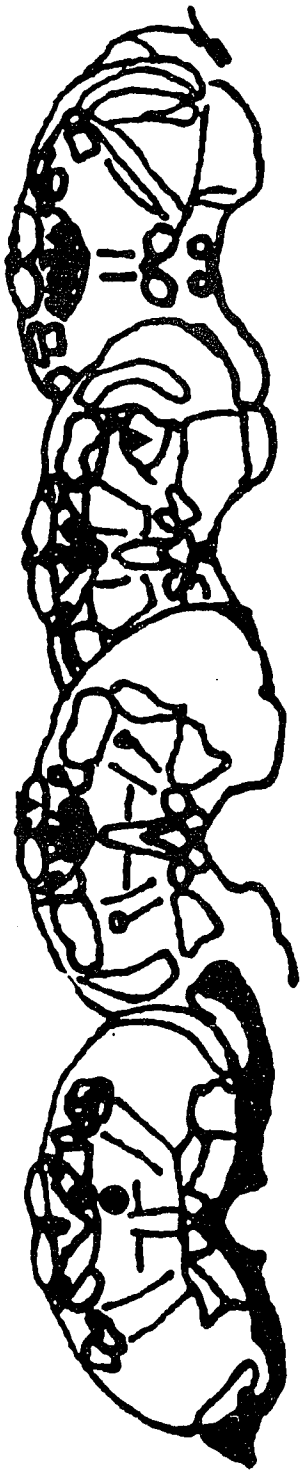
caudal as the nucleus of the VII cranial nerve (Paxinos and Watson, 1986), sites which have been implicated in opioid antinociception (e.g., Azami et al., 1986; Satoh et al., 1983). In the remaining 3 rats, medullary placements were outside of these areas (Figure 1), and their data were grouped separately.

Table 1. Summary of experimental groups in the methysergide protocol.

RVM Condition	PAG Condition	n
Vehicle	Vehicle	17
Vehicle	Morphine (2.5 μg)	17
Methysergide (0.5 μg)	Morphine (2.5 μg)	3
Methysergide (2 μg)	Morphine (2.5 μg)	3
Methysergide (5 μg)	Morphine (2.5 μg)	14
Methysergide (10 μg)	Vehicle	6

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

Figure 1. Histological verification of cannula placements using the atlas of Paxinos and Watson (1986). The upper panel depicts mesencephalic cannula placements (Bregma: -6.04 mm to -7.64 mm) in which morphine (2.5 μg) elicited an antinociceptive response on the tail-flick and jump tests. The lower panel depicts medullary cannula placements (Bregma: -10.04 mm to -11.60 mm) in which methysergide (0.5-5 μg) was administered 15 min prior to the opiate. Closed circles depict mesencephalic and medullary placements of rats with medullary cannulae in the RVM. Closed triangles depict mesencephalic and medullary placements of rats with medullary cannulae lateral or ventral to those structures.



Significant differences were found among conditions (tail-flick: $F(6,53)= 9.28$, $p<.001$; jump: $F= 5.84$, $p<.001$), across the time course (tail-flick: $F(3,159)= 20.19$, $p<.001$; jump: $F= 2.98$, $p<.033$) and for the interaction between conditions and times (tail-flick: $F(18,159)= 4.52$, $p<.001$; jump: $F= 3.55$, $p<.001$). Figure 2 illustrates the significant morphine antinociception following PAG microinjections relative to vehicle controls on the tail-flick (upper left panel) and jump (upper right panel) tests. Methysergide (5 μg) microinjected into the RVM significantly reduced morphine antinociception elicited from the PAG on the tail-flick test at 30 (66%), 60 (69%) and 90 (68%) min, and on the jump test at 30 (48%) and 60 (50%) min. Methysergide (5 μg) microinjected into the RVM failed to alter basal latencies or thresholds. If methysergide was microinjected into sites that were either lateral or ventral to the RVM (Figure 1, lower panel), it failed to alter morphine antinociception elicited from the PAG on the tail-flick test (overall 7% increase), and only transiently reduced morphine antinociception on the jump test at 30 min (40%). The overall reduction of morphine antinociception on the jump test was smaller in these control sites (15%) relative to RVM sites (38%). The reductions in PAG morphine antinociception on the tail-flick (Figure 2, lower left panel) and jump (Figure 2, lower right panel) tests by methysergide administered into the RVM were dose-dependent with a 2 μg dose significantly reducing morphine

antinociception on the tail-flick (30 and 60 min; 42-45%) and jump (30 and 60 min; 49-56%) tests, and a 0.5 μg dose significantly reducing morphine antinociception on the tail-flick (30-90 min; 45-87%) and jump (30-90 min; 41-67%) tests.

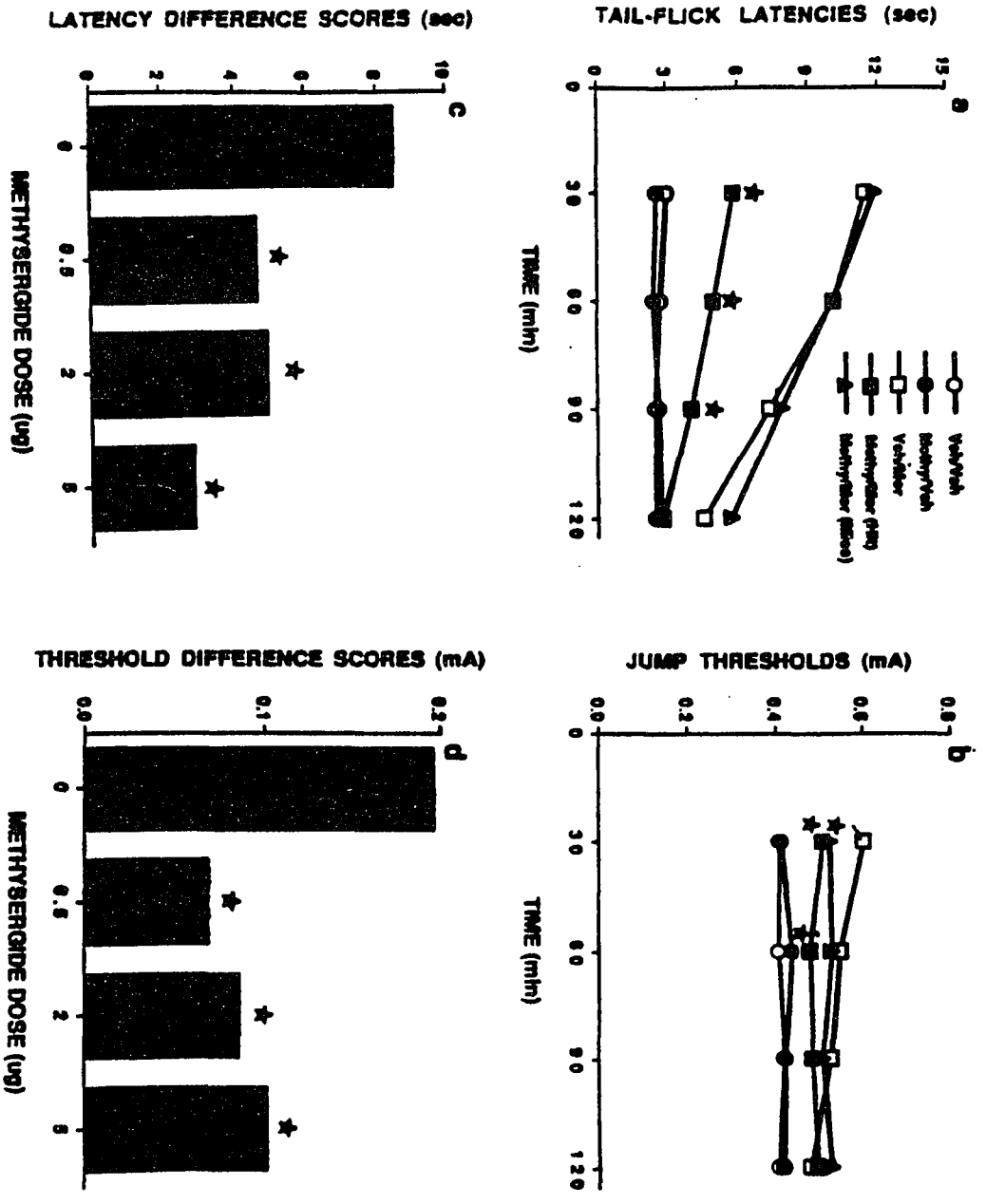
Discussion

The present study found that methysergide microinjected into the RVM produces a dose-dependent reduction in morphine antinociception elicited from the PAG. This effect is site-specific since methysergide administered into medullary loci either lateral or ventral to these structures failed to alter mesencephalic morphine antinociception. Further, these effects are not due to an intrinsic action by methysergide upon basal nociception since methysergide microinjected into the RVM failed to alter either basal tail-flick latencies or jump thresholds.

The proposal that an endogenous supraspinal opioid pain-inhibitory system originated in the midbrain PAG, synapsed in the NRM and projected to the substantia gelatinosa of the spinal cord through the dorsolateral funiculus (Basbaum and Fields, 1984; Fields and Basbaum, 1978) has been supported by the physiological characterization of medullary neurons mediating supraspinal antinociception (Barbaro et al., 1986; Morgan, Sohn and Liebeskind, 1989; Sandkuhler and Gebhart, 1984a,b; Vanegas et al., 1984; Zhuo and Gebhart, 1990; Zorman et al., 1981). The direct connections from the PAG to the NRM and the NRGC (Abols and Basbaum, 1981; Beitz et al., 1983;

Gallagher and Pert, 1978; VanBockstaele et al., 1989) contain serotonin as one of their transmitters (Beitz, 1982a). This finding, together with the localization of serotonin receptors on medial medullary neurons (Pazos et al., 1985a,b) indicates that serotonin is a serious candidate for PAG-RVM communication with respect to antinociceptive processes. The present data confirms the hypothesis that a serotonergic synapse participates in the PAG-medial medullary pathway mediating the activation of mesencephalic opioid receptors by either endogenous or exogenous opioids as well as exogenous opiates. The ability of methysergide administered into the RVM to block morphine antinociception elicited from the PAG confirms the participation of serotonin in the modulation of supraspinal opioid antinociception in the medial medulla, and confirms the postulated supraspinal circuitry through which opioids act to produce antinociception (Basbaum and Fields, 1984; Fields and Basbaum, 1978).

Figure 2. Alterations in tail-flick latencies (a & c) and jump thresholds (b & d) in rats microinjected with vehicle (Veh) or morphine (Mor, 2.5 μg) in the mesencephalic periaqueductal gray region 15 min after pretreatment with Veh or methysergide (Methy, 5 μg: a & b) microinjected into the RVM (Hit group) or into medullary structures lateral or ventral to these loci (Miss group). Morphine significantly increased latencies (30-90 min) and thresholds (30-120 min) (Dunnett comparisons, $p<.05$). The stars denote significant reductions in morphine antinociception by methysergide (Dunn comparisons, $p<.05$). Panels c and d illustrate significant reductions in the peak (30 min) magnitude of morphine antinociception by a dose range of methysergide using difference scores which were derived by subtracting the vehicle score from each respective experimental score.


LATENCY DIFFERENCE SCORES (sec)
METHYSERGIDE DOSE (ug)
TAIL-FLICK LATENCIES (sec)
TIME (min)
THRESHOLD DIFFERENCE SCORES (mA)
METHYSERGIDE DOSE (ug)
JUMP THRESHOLDS (mA)
TIME (min)

EXPERIMENT 2: RVM RITANSERIN AND ICS 205930/PAG MORPHINE ANTINOCICEPTION

The first experiment (Kiefel et al., 1991) investigated whether a serotonergic medullary synapse mediates morphine antinociception elicited from the PAG and found that methysergide administered into the RVM significantly reduced mesencephalic morphine antinociception on the tail-flick and jump tests. Importantly, methysergide in the RVM failed to alter nociceptive thresholds itself, and methysergide microinjected into sites dorsal, lateral or ventral to the RVM failed to affect mesencephalic morphine antinociception.

Multiple serotonin receptors include the 5-HT_2 and 5-HT_3 subtypes (e.g., see reviews: Peroutka 1988a,b; Peroutka et al., 1990) which are found on medial medullary neurons (Pazos et al., 1984; 1985; Waeber et al., 1988). Both 5-HT_2 and the 5-HT_3 receptor subtypes have been implicated in the mediation of opioid antinociception (Kiefel et al., 1989; Paul et al., 1986; 1988; 1990; Ho and Takemori, 1990). To assess whether medullary 5-HT_2 and 5-HT_3 receptor subtypes mediate morphine antinociception elicited from the PAG, this experiment determined whether microinjections of either the 5-HT_2 receptor antagonist, ritanserin or the 5-HT_3 receptor antagonist, ICS 205930 into the RVM would alter the antinociception elicited by morphine microinjections into the PAG.

Protocol

After surgery and determination of baseline measures, each rat received pairs of microinjection conditions at weekly intervals. Tail-flick latencies and jump thresholds were again determined at 30, 60, 90 and 120 min following the second microinjection of each pair. Table 2 summarizes the experimental treatments, doses and sample sizes used to assess the effects of medullary ritanserin (A) and ICS 205930 (B) upon morphine antinociception elicited from the PAG. All medullary microinjections preceded mesencephalic microinjections by 15 min to allow 5-HT subtype antagonist effects to develop (Beczkowska, Koch and Bodnar, 1992a).

Results

Mesencephalic cannula placements were all localized in the lateral, ventral and ventrolateral quadrants of the PAG and immediately-adjacent tegmentum as far rostral as the III cranial nerve nucleus and as far caudal as the dorsal raphe nucleus; a representative mesencephalic cannula placement in the ventrolateral quadrant of the PAG is illustrated in Figure 3A. Each of these placements were capable of eliciting morphine antinociception. In 20 rats, medullary cannula placements were localized in either the NRM, NRGC or 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; a representative cannula placement in the RVM is illustrated in Figure 3B. In the remaining five rats, medullary placements

were outside of the RVM and included such structures as the pyramidal tract and the nucleus of the VIIth cranial nerve (see Figure 3C). The data of the latter rats were grouped separately. Significant differences were found among ritanserin-morphine conditions (tail-flick: $F(5,30)= 8.76$, $p<.001$; jump: $F= 7.15$, $p<.001$), across the time course (tail-flick: $F(3,90)= 21.45$, $p<.001$) and for the interaction between conditions and times (tail-flick: $F(15,90)= 5.10$, $p<.001$; jump: $F= 1.99$, $p<.025$). Morphine in the PAG significantly increased tail-flick latencies (30-90 min, Figure 4A) and jump thresholds (30-120 min, Figure 4B) which were significantly reduced by ritanserin (2.5 μg) pretreatment in the RVM. Ritanserin microinjections into the RVM failed to alter basal latencies or thresholds. Peak morphine antinociception was significantly and dose-dependently reduced by ritanserin on the tail-flick (Figure 4C: 2.5 μg (81%), 1.25 μg (34%), 0.25 μg (7% increase)) and jump (Figure 4D: 2.5 μg (65%), 1.25 μg (43%), 0.25 μg (11% increase)) tests. Total morphine antinociception was also significantly reduced by ritanserin on the tail-flick (Figure 4E: 2.5 μg (80%), 1.25 μg (67%), 0.25 μg (17% increase)) and jump (Figure 4F: 2.5 μg (61%), 1.25 μg (34%), 0.25 μg (5% increase)) tests.

Significant differences were found among ICS 205930-morphine conditions (tail-flick: $F(5,38)= 8.88$, $p<.001$; jump: $F= 5.84$, $p<.001$), across the time course (tail-flick: $F(3,114)= 16.34$, $p<.001$) and for the interaction between

conditions and times (tail-flick: $F(15,114)= 7.07$, $p<.001$). Morphine in the PAG significantly increased tail-flick latencies (Figure 5A) and jump thresholds (Figure 5B) which were significantly reduced by ICS 205930 (5 μg) pretreatment in the RVM. ICS 205930 microinjections into the RVM failed to alter basal latencies or thresholds. Peak morphine antinociception was significantly and dose-dependently reduced by ICS 205930 on the tail-flick (Figure 5C: 5 μg (91%), 1 μg (27%), 0.25 μg (59%)) and jump (Figure 5D: 5 μg (63%), 1 μg (47%), 0.25 μg (77%)) tests. Total morphine antinociception was also significantly reduced by ICS 205930 on the tail-flick (Figure 5E: 5 μg (88%), 1 μg (30%), 0.25 μg (14%)) and jump (Figure 5F: 5 μg (61%), 1 μg (35%), 0.25 μg (47%)) tests.

If ritanserin or ICS 205930 were microinjected into sites that were either lateral or ventral to the RVM, significant differences were found among conditions (tail-flick: $F(3,11)= 6.94$, $p<.007$; jump: $F= 6.25$, $p<.010$), across the time course (tail-flick: $F(3,33)= 29.45$, $p<.001$; jump: $F= 7.98$, $p<.001$) and for the interaction between conditions and times (tail-flick: $F(9,33)= 5.55$, $p<.001$; jump: $F= 3.52$, $p<.004$). In rats with misplaced medullary placements, neither ritanserin (7% decrease) nor ICS 205930 (18% increase) significantly altered mesencephalic morphine antinociception on the tail-flick test (Table 3). However, in rats with misplaced medullary placements, ritanserin significantly reduced by 49% mesencephalic morphine antinociception on the jump test at 90

and 120 min after the injection. In contrast, misplaced injections of ICS 205930 significantly increased mesencephalic morphine antinociception on the jump test after 30 and 60 min, and decreased it after 90 min (Table 3). Misplaced injections of ritanserin and ICS 205930 failed to alter basal nociceptive thresholds (data not shown).

Discussion

The present study has implicated the 5-HT_2 and the 5-HT_3 receptor subtypes in the modulation of mesencephalic morphine antinociception since microinjections of either ritanserin or ICS 205930 into the RVM dose dependently reduced morphine antinociception elicited from the PAG. These effects occurred in the absence of any intrinsic effect of these antagonists upon basal nociceptive latencies and thresholds. Microinjections of ritanserin and ICS 205930 into medullary sites ventral or lateral to the RVM failed to alter mesencephalic morphine antinociception on the tail-flick test. Whereas misplaced medullary injections of ICS 205930 generally failed to affect mesencephalic morphine antinociception on the jump test, misplaced medullary ritanserin injections reduced mesencephalic morphine antinociception on this nociceptive measure after 90-120 min. This effect was smaller in magnitude and delayed in onset relative to properly placed ritanserin microinjections. It is possible that diffusion of the antagonist to the proper sites produced this delayed and diminished response. The present data, together with the

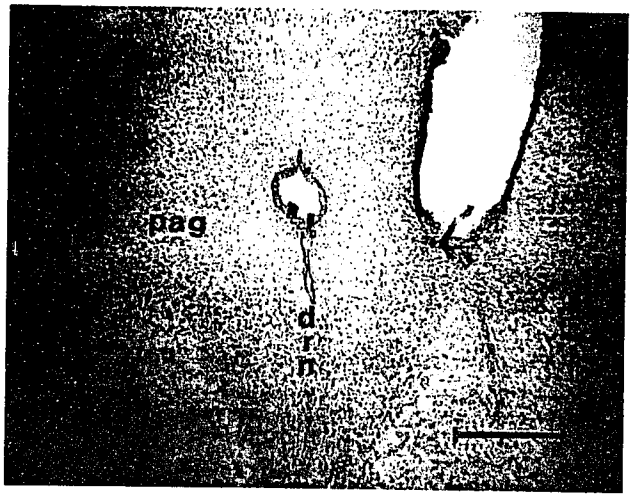
existence of dense 5-HT_2 and 5-HT_3 binding on medial medullary neurons (Pazos et al., 1984;1985; Waeber et al., 1988) suggests that mesencephalic morphine may activate antinociceptive circuits in the RVM in part through a serotonergic PAG-RVM pathway which utilizes 5-HT_2 and 5-HT_3 receptor subtypes. Moreover, both ritanserin and ICS 205930 produced greater inhibitory effects across the time course than methysergide. It is possible that methysergide might be inhibiting another 5-HT subtype that antagonizes 5-HT_2 and 5-HT_3 effects. It might be interesting to examine the effects of co-administration of 5-HT_2 and 5-HT_3 antagonists, and see whether this might produce a total inhibition of morphine's antinociceptive effects. However, without having potent and selective ligands for particular receptor subtypes, such research is limited in terms of its interpretation.

Table 2. Summary of experimental groups in the ritanserin and ICS 205930 protocols.

RVM Condition	PAG Condition	n
A. Ritanserin Group:		
Vehicle		12
Vehicle	Morphine (2.5 μg)	12
Ritanserin (0.25 μg)	Morphine (2.5 μg)	2
Ritanserin (1.25 μg)	Morphine (2.5 μg)	4
Ritanserin (2.5 μg)	Morphine (2.5 μg)	10
Ritanserin (2.5 μg)	Vehicle	5
B. ICS 205930 Group:		
Vehicle	Vehicle	13
Vehicle	Morphine (2.5 μg)	13
ICS205930 (0.25 μg)	Morphine (2.5 μg)	4
ICS205930 (1 μg)	Morphine (2.5 μg)	5
ICS205930 (5 μg)	Morphine (2.5 μg)	11
ICS205930 (5 μg)	Vehicle	4

Figure 3. A. Representative photomicrograph of a mesencephalic cannula placement in the ventrolateral quadrant of the PAG which elicited antinociception following morphine microinjections. B. Representative photomicrograph of a medullary cannula placement in the RVM through which ritanserin significantly reduced mesencephalic morphine antinociception. C. Representative photomicrograph of a medullary cannula placement in the nucleus of seventh cranial nerve (VII) through which ritanserin failed to alter mesencephalic morphine antinociception.

drn: dorsal raphe nucleus, pyr: pyramidal tract. Scale bar: 500 um.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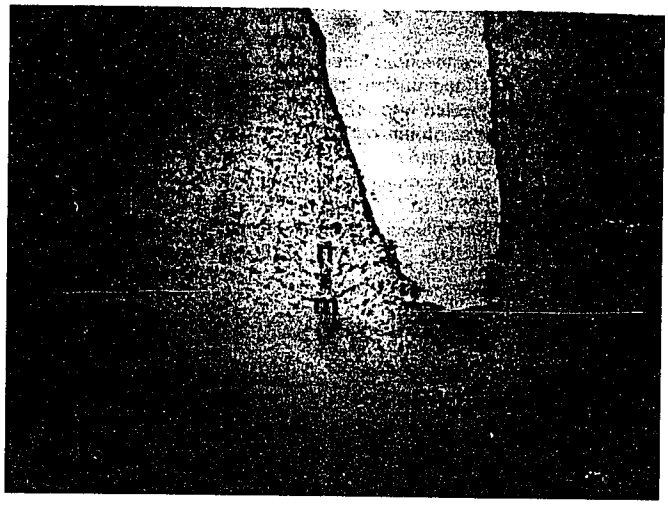
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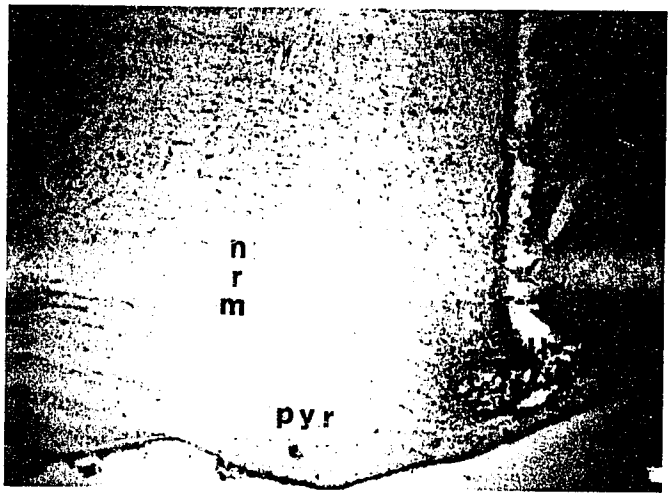
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Figure 4. Alterations in tail-flick latencies (A,C,E) and jump thresholds (B,D,F) in rats microinjected with vehicle (solid circles) or morphine (2.5 μg) in the mesencephalic PAG region 15 min after pretreatment with Veh (solid triangles) or ritanserin (2.5 μg, solid squares) microinjected into the RVM. The significantly increased latencies (30-90 min) and thresholds (30-120 min) following morphine were significantly reduced by ritanserin (solid stars). Ritanserin failed to alter basal latencies or thresholds (solid diamonds). Significant reductions in peak (Panels C,D: 30 min) and total (Panels E,F) magnitudes of morphine antinociception were produced by a dose range (0.25, 1.25 and 2.5 μg) of ritanserin using difference scores which were derived by subtracting the vehicle score from each respective experimental score.

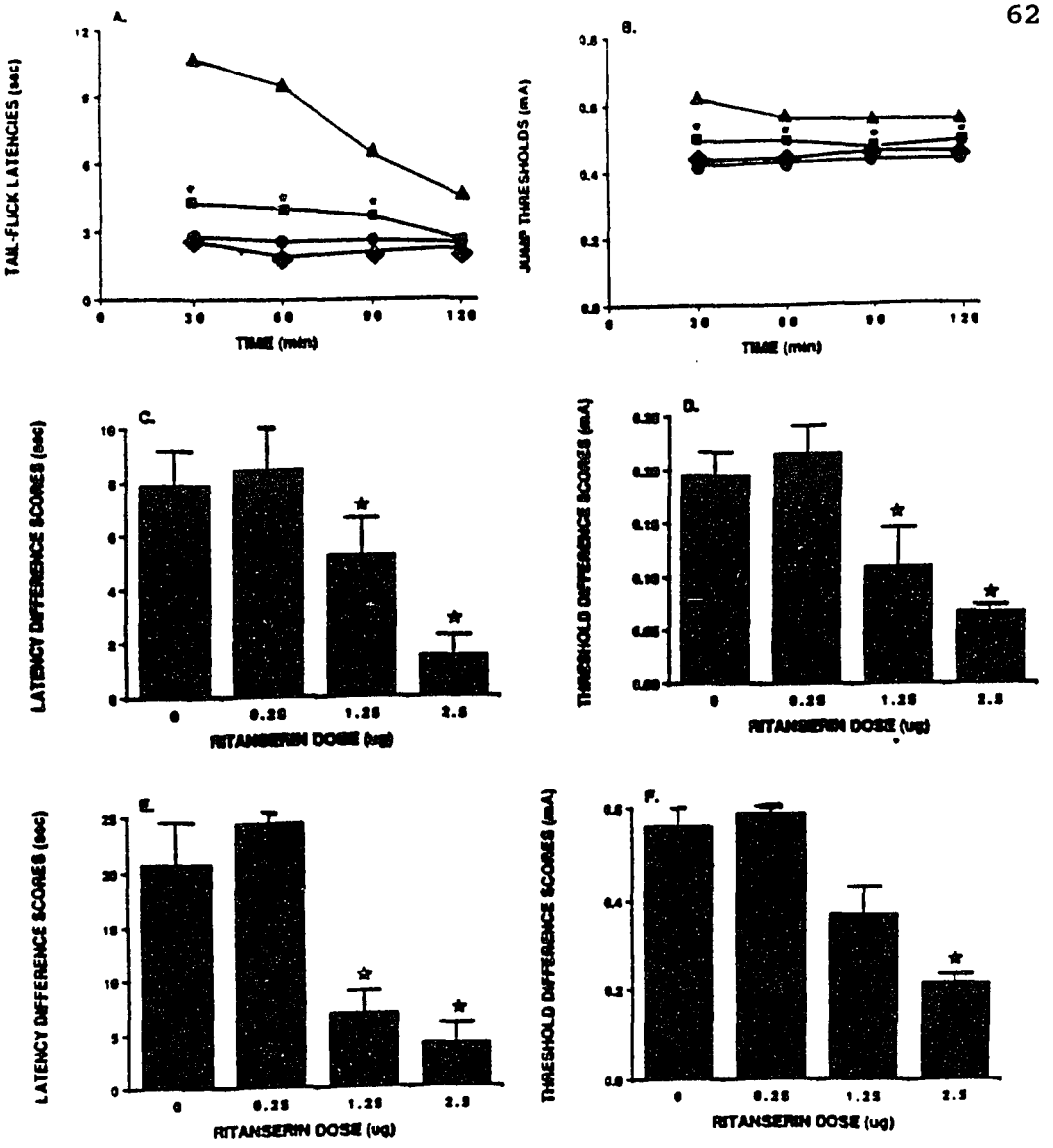

A.
TAIL-FLICK LATENCIES (sec)
TIME (min)
B.
JUMP THRESHOLDS (mA)
TIME (min)
C.
LATENCY DIFFERENCE SCORES (sec)
RITANSERIN DOSE (ug)
D.
THRESHOLD DIFFERENCE SCORES (mA)
RITANSERIN DOSE (ug)
E.
LATENCY DIFFERENCE SCORES (sec)
RITANSERIN DOSE (ug)
F.
THRESHOLD DIFFERENCE SCORES (mA)
RITANSERIN DOSE (ug)

Figure 5. Alterations in tail-flick latencies (A,C,E) and jump thresholds (B,D,F) in rats microinjected with vehicle (solid circles) or morphine (2.5 μg) in the mesencephalic PAG region 15 min after pretreatment with Veh (solid triangles) or ICS 205930 (5 μg, solid squares) microinjected into the RVM. The significantly increased latencies (30-90 min) and thresholds (30-120 min) following morphine were significantly reduced by ICS 205930 (solid stars). ICS 205930 failed to alter basal latencies or thresholds (solid diamonds). Significant reductions in peak (Panels C,D) and total (Panels E,F) magnitudes of morphine antinociception were produced by a dose range (0.25, 1, 5 μg) of ICS 205930 using difference score analyses.

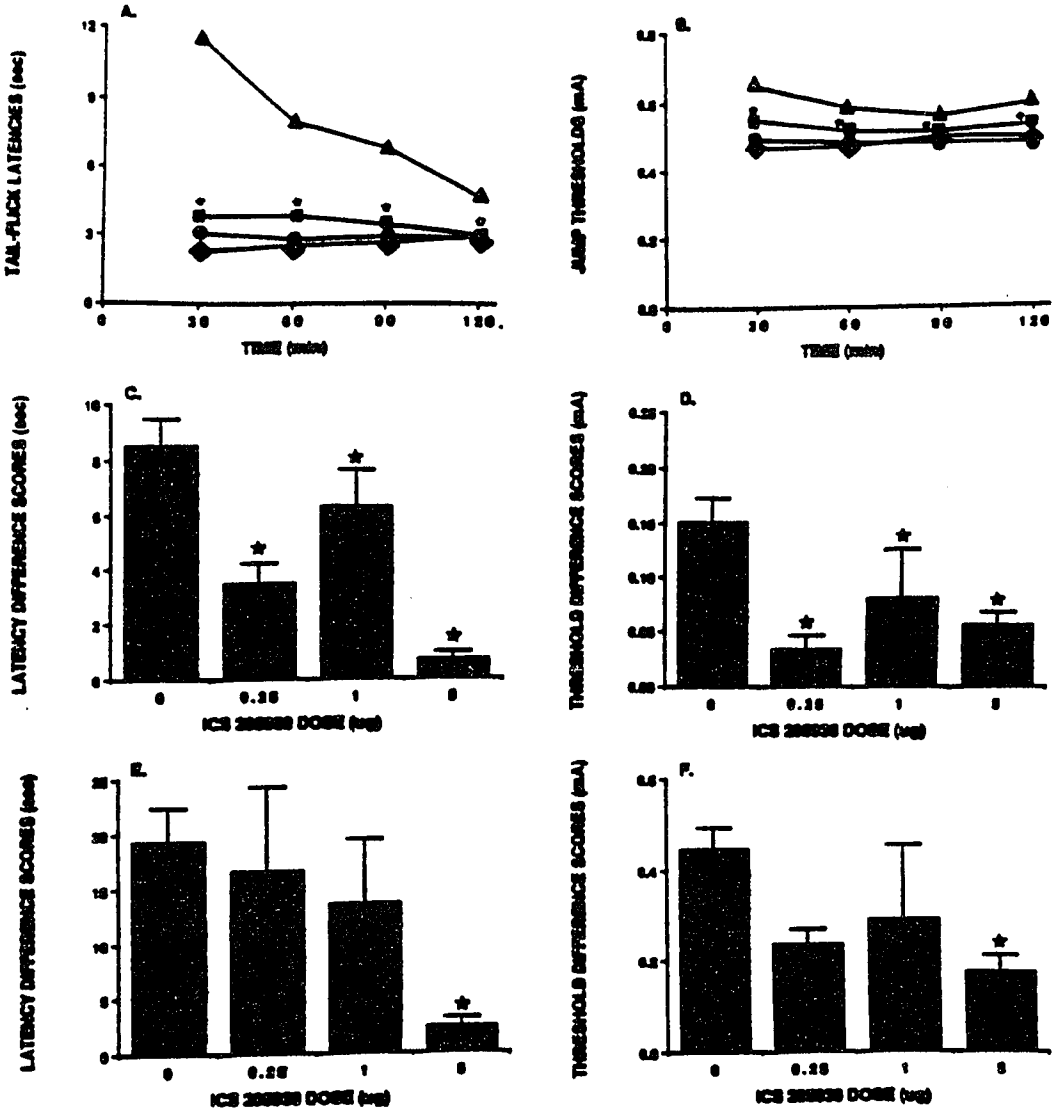

A.
TAIL-FLICK LATENCIES (sec)
TIME (min)
B.
JUMP THRESHOLDS (mA)
TIME (min)
C.
LATENCY DIFFERENCE SCORES (sec)
ICS 205930 DOSE (mg)
D.
THRESHOLD DIFFERENCE SCORES (mA)
ICS 205930 DOSE (mg)
E.
LATENCY DIFFERENCE SCORES (sec)
ICS 205930 DOSE (mg)
F.
THRESHOLD DIFFERENCE SCORES (mA)
ICS 205930 DOSE (mg)

Table 3. Summary of ritanserin (2.5 μg) and ICS 205930 (5 μg) effects upon mesencephalic morphine (2.5 μg) antinociception in rats with misplaced medullary cannulae.

GROUP	TAIL-FLICK LATENCIES				JUMP THRESHOLDS			
	30	60	90	120	30	60	90	120
Veh/Veh	3.00	3.01	2.78	3.06	.470	.476	.478	.478
Veh/Mor	12.90	11.19	6.11	4.31	.590	.595	.588	.581
Rit/Mor	13.78	8.89	5.35	4.99	.550	.550	.517*	.519*
ICS/Mor	12.28	13.90	5.34	7.16	.675*	.666*	.521*	.550

*Significantly different from corresponding Veh/Mor value (Dunn Comparison, $p<.05$).

EXPERIMENT 3: RVM NALTREXONE, BETA-FUNALTREXAMINE AND NALTRINDOLE/PAG MORPHINE ANTINOCICEPTION

The first two studies (Kiefel et al., 1991, 1992) examined whether serotonergic medullary synapses participated in the mediation of morphine antinociception elicited from the PAG and found that pretreatment in the RVM with either the general 5-HT antagonist, methysergide, the 5-HT_2 antagonist, ritanserin, or the 5-HT_3 antagonist, ICS 205930 significantly reduced mesencephalic morphine antinociception on both the tail-flick and jump tests. None of the antagonists altered nociceptive latencies or thresholds themselves, and all antagonists failed to significantly alter mesencephalic morphine antinociception when medullary cannula placements were either lateral, dorsal or ventral to the RVM.

Enkephalinergic-immunoreactive neurons project from the PAG to the RVM (Beitz, 1982b), and also serve as intrinsic interneurons in the RVM (Lewis et al., 1985). Both mu and delta opiate receptors have been localized in each of these nuclei (Atweh and Kuhar, 1977a,b; Goodman et al., 1980; Lewis et al., 1980, 1985; Moskowitz and Goodman, 1985a,b). Both mu and delta agonists produce antinociception following supraspinal and spinal administration (e.g., Azami et al., 1982; Bodnar et al., 1988; Satoh et al., 1983; Zorman et al., 1981). In addition, naltrexone, ß-FNA and naltrindole have been shown to be effective in antinociceptive assays (e.g., Jiang et al., 1990b; Paul et al., 1989; Sofuoglu et al., 1991;

Takemori et al., 1981; Ward et al., 1982, 1983). To ascertain whether an opioid synapse in the RVM participates in the mediation of morphine antinociception elicited from the PAG, this study examined whether mesencephalic morphine antinociception on the tail-flick and jump tests was altered following pretreatment in the RVM of either naltrexone, ß-FNA or naltrindole. Further, to ascertain the anatomical specificity of such effects, peak doses of each antagonist were administered into sites lateral and dorsal to the RVM to determine their actions upon mesencephalic morphine antinociception.

Protocol

After surgery and determination of baseline measures, each rat received pairs of microinjection conditions at weekly intervals. Latencies and thresholds were assessed 30, 60, 90 and 120 min following the second microinjection. Table 4 summarizes the experimental treatments, doses and sample sizes used to assess the effects of naltrexone (A), ß-FNA (B) or naltrindole (C) administered into the RVM upon mesencephalic morphine antinociception. Table 4D also summarizes the experimental treatments of the control rats with misplaced medullary cannulae. Medullary microinjections preceded mesencephalic microinjections by 15 min in the naltrexone and naltrindole protocols to allow antagonist effects to develop (Pasternak and Wood, 1986; Zukin and Zukin, 1981; Drower, Stapelfeld, Raferty, DeCosta, Rice and Hammond, 1991;

Portoghese et al., 1988a,b), and by 24 h in the ß-FNA protocol to allow its irreversible mu-selective actions to develop (Portoghese et al., 1980; Takemori et al., 1981).

Table 4. Summary of experimental groups in the opioid antagonist protocols.

RVM Condition	PAG Condition	n
A. Naltrexone Group:		
Vehicle	Vehicle	17
Vehicle	Morphine (2.5 μg)	17
Naltrexone (1 μg)	Morphine (2.5 μg)	5
Naltrexone (2.5 μg)	Morphine (2.5 μg)	5
Naltrexone (5 μg)	Morphine (2.5 μg)	6
Naltrexone (10 μg)	Morphine (2.5 μg)	6
Naltrexone (10 μg)	Vehicle	3
B. Beta-funaltrexamine (ß-FNA) Group:		
Vehicle	Vehicle	5
Vehicle	Morphine (2.5 μg)	5
ß-FNA (0.5 μg)	Morphine (2.5 μg)	4
ß-FNA (5 μg)	Morphine (2.5 μg)	5
C. Naltrindole Group:		
Vehicle	Vehicle	9
Vehicle	Morphine (2.5 μg)	9
Naltrindole (0.5 μg)	Morphine (2.5 μg)	5
Naltrindole (5 μg)	Morphine (2.5 μg)	6
D. Control Group:		
Vehicle	Vehicle	9
Vehicle	Morphine (2.5 μg)	9
Naltrexone (5 μg)	Morphine (2.5 μg)	6
ß-FNA (5 μg)	Morphine (2.5 μg)	6
Naltrindole (5 μg)	Morphine (2.5 μg)	6

Note: The control group had medullary placements that were lateral or dorsal to the RVM. RVM = rostral ventral medulla; PAG = periaqueductal gray.

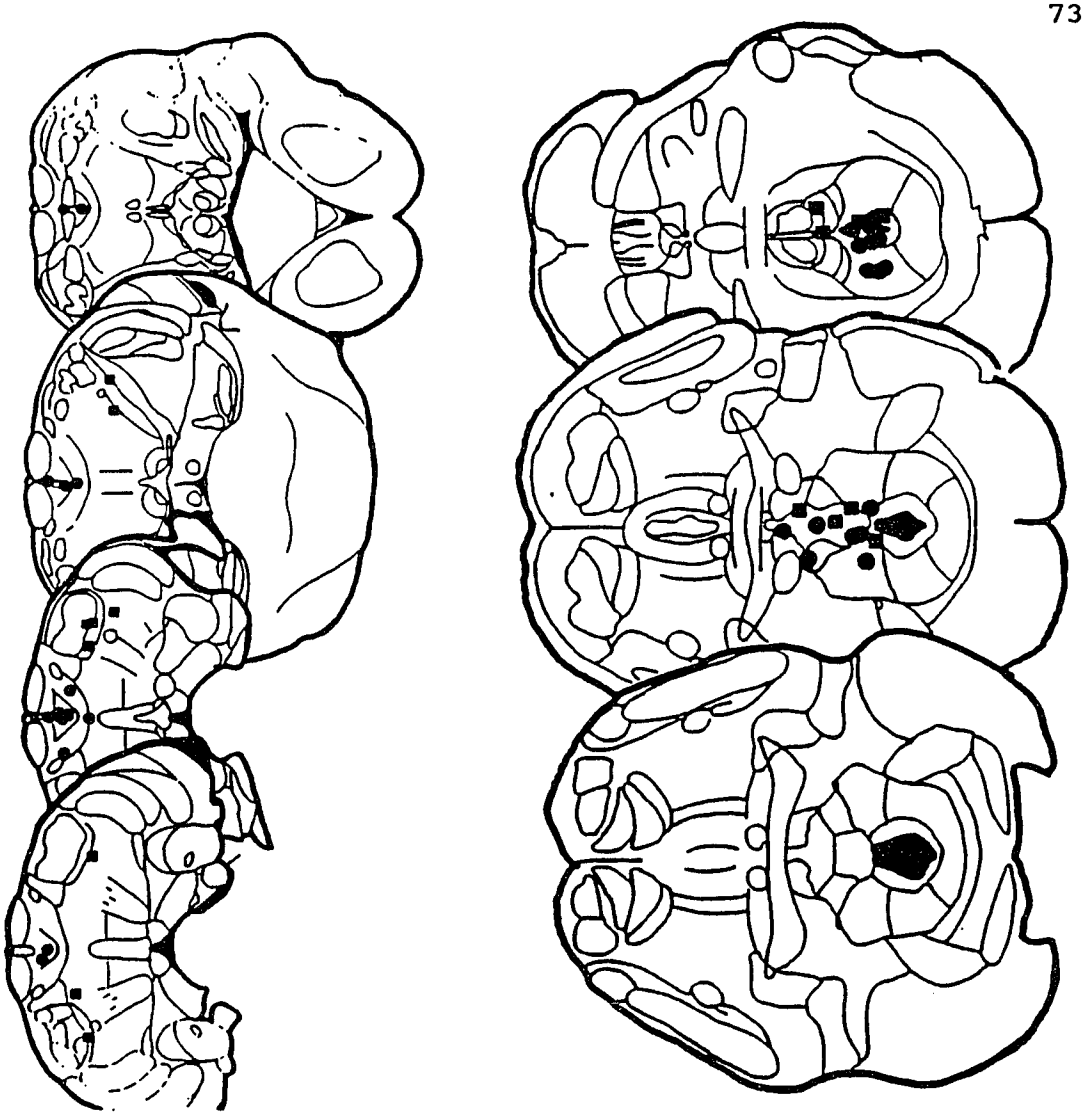
Results

Mesencephalic cannula placements were all localized in the lateral, ventral and ventrolateral quadrants of the PAG and immediately-adjacent tegmentum as far rostral as the III cranial nerve nucleus and as far caudal as the dorsal raphe nucleus (Figure 6, upper panel). RVM cannula placements were localized in either the NRM, NRGC or 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 (closed circles in Figure 6, lower panel). Control medullary cannula placements were localized along the rostro-caudal extent of RVM placements, but were located lateral and dorsal to this area (closed squares in Figure 6, lower panel). It should be noted that there was considerable anatomical overlap in the location of the mesencephalic cannula placements of rats with RVM (closed circles) and control medullary (closed squares) cannula placements (Figure 6). Further, the magnitude of mesencephalic morphine antinociception was similar in rats with RVM placements and control medullary placements, and was similar in rostral and caudal PAG placements. The latter result is in agreement with other recent data from our laboratory (Robertson and Bodnar, 1993).

RVM naltrexone and PAG morphine antinociception: Significant differences were found among the naltrexone-morphine conditions (tail-flick: $F(6,52) = 27.88$, $p<.001$; jump: $F = 6.63$, $p<.001$), across the time course (tail-flick:

$F(3,156)= 13.27$, $p<.001$) and for the interaction between conditions and times (tail-flick: $F(18,156)= 6.09$, $p<.001$; jump: $F= 2.85$, $p<.001$).

Figure 6. Histological verification of cannula placements using the atlas of Paxinos and Watson (1986). The upper panel depicts mesencephalic cannula placements in which morphine (2.5 μg) elicited an antinociceptive response on the tail-flick and jump tests (Figures 45, 48 and 50 in 50). The lower panel depicts medullary cannula placements in which the different opioid antagonists were administered prior to the opiate (Figures 55, 58, 61 and 63 in 50). Closed circles depict mesencephalic and medullary placements of rats with medullary cannulae in the RVM. Closed squares depict mesencephalic and medullary placements of rats with medullary cannulae lateral or dorsal to those structures.

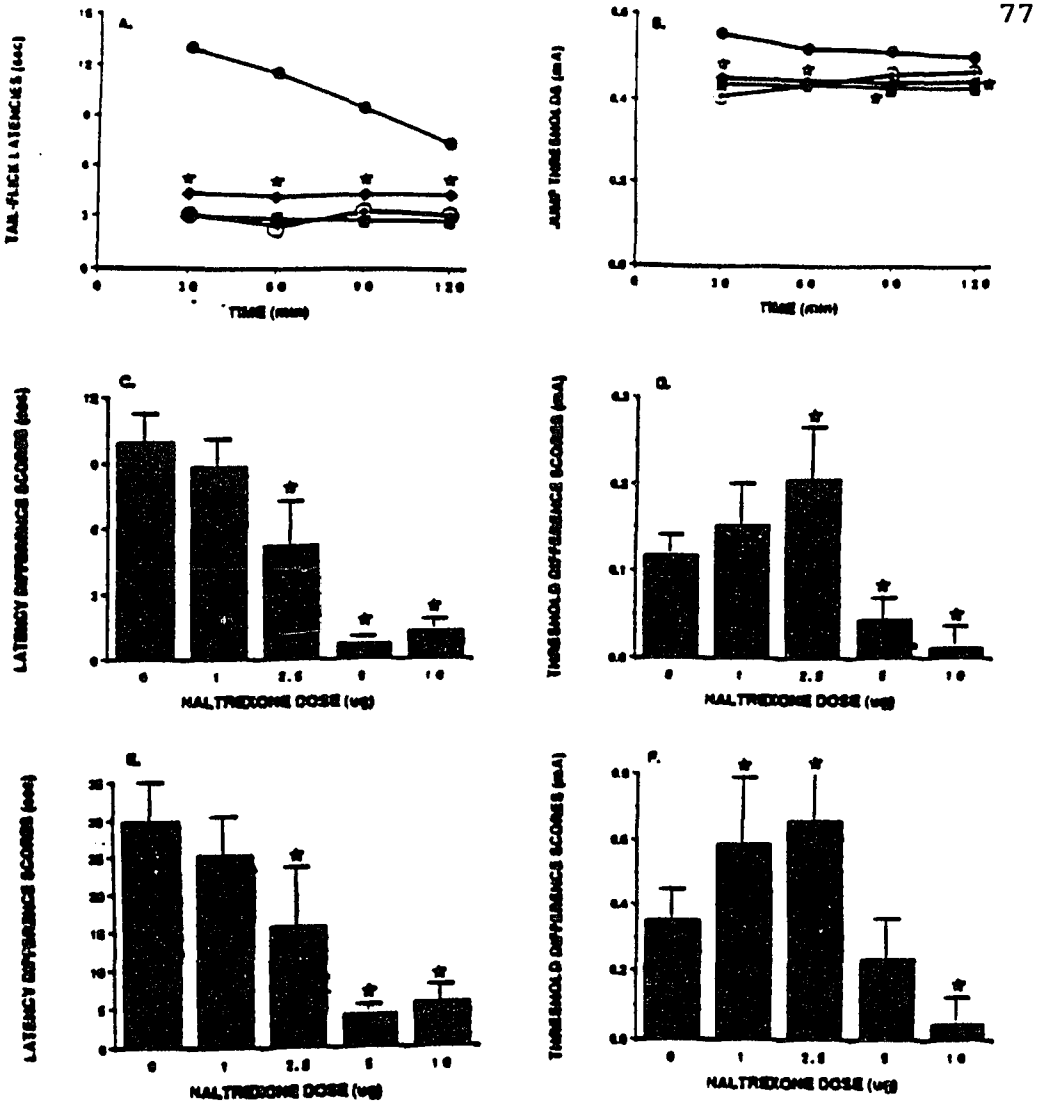


Morphine in the PAG significantly increased tail-flick latencies (Figure 7A) and jump thresholds (Figure 7B) across the time course; pretreatment with the 10 μg dose of naltrexone in the RVM blocked these antinociceptive responses across the time course. Naltrexone microinjections in the RVM failed to alter basal latencies or thresholds. The dose-dependent effects of naltrexone in the RVM upon the magnitude of mesencephalic morphine antinociception varied as a function of the nociceptive test. On the tail-flick test, mesencephalic morphine antinociception was significantly and dose-dependently reduced by naltrexone doses of 2.5 (peak: 48%; total: 48%), 5 (peak: 93%; total: 86%) and 10 (peak: 87%; total: 81%) μg in the RVM (Figures 7C and 7E). The 1 μg dose of naltrexone in the RVM nonsignificantly reduced peak (11%) and total (15%) antinociceptive effects on the tail-flick test. On the jump test, mesencephalic morphine antinociception was significantly and dose-dependently reduced by naltrexone doses of 5 (peak: 62%) and 10 (peak: 89%; total: 86%) μg in the RVM (Figures 7D and 7F). The 5 μg naltrexone dose in the RVM failed to significantly alter total (32% decrease) antinociceptive effects on the jump test. In contrast, mesencephalic morphine antinociception on the jump test was significantly increased by naltrexone doses of 1 (total: 67%) and 2.5 (peak: 75%; total: 88%) μg in the RVM.

RVM ß-FNA and PAG morphine antinociception: Significant differences were found among the ß-FNA-morphine conditions

(tail-flick: $F(3,15)= 16.71$, $p<.001$; jump: $F= 8.41$, $p<.002$), across the time course (tail-flick: $F(3,45)= 8.68$, $p<.001$) and for the

Figure 7. Alterations in tail-flick latencies (A,C,E) and jump thresholds (B,D,F) in rats microinjected with vehicle (solid squares) or morphine (2.5 μg) in the mesencephalic PAG region 15 min after pretreatment with vehicle (solid circles) or naltrexone (10 μg, solid diamonds) microinjected into the RVM. The significantly increased latencies and thresholds across the time course following morphine were significantly reduced by naltrexone (open stars). Naltrexone failed to alter basal latencies or thresholds (open circles). Significant reductions in peak (Panels C,D: 30 min) and total (Panels E,F) magnitudes of morphine antinociception were produced by a dose range (1-10 μg) of naltrexone using difference scores which were derived by subtracting the vehicle score from each respective experimental score.


A.
B.
C.
D.
E.
F.
TIME (min)
NALTREXONE DOSE (ug)

interaction between conditions and times (tail-flick: $F(9,45)= 2.71$, $p<.013$). Morphine in the PAG significantly increased latencies (Figure 8A) and thresholds (Figure 8B) across the time course; pretreatment with the 5 μg dose of ß-FNA in the RVM blocked these antinociceptive responses across the time course. The magnitude of mesencephalic morphine antinociception was significantly and dose-dependently reduced by both ß-FNA doses of 0.5 (peak: 65%; total: 57%) and 5 (peak: 93%; total: 94%) μg in the RVM on the tail-flick test (Figures 8C and 8E), and by both ß-FNA doses of 0.5 (peak: 56%) and 5 (peak: 91%; total: 80%) μg in the RVM on the jump test (Figures 8D and 8F).

RVM naltrindole and PAG morphine antinociception: Significant differences were found among the naltrindole-morphine conditions (tail-flick: $F(3,25)= 46.52$, $p<.001$; jump: $F= 4.70$, $p<.01$), across the time course (tail-flick: $F(3,75)= 14.19$, $p<.001$; jump: $F= 2.61$, $p<.058$) and for the interaction between conditions and times (tail-flick: $F(9,75)= 3.55$, $p<.001$). Morphine in the PAG significantly increased latencies (Figure 9A) and thresholds (Figure 9B) across the time course; pretreatment with the 5 μg dose of naltrindole in the RVM blocked morphine antinociception on the tail-flick test across the time course, and on the jump test at only 30 min after the injection. The dose-dependent effects of naltrindole in the RVM upon the magnitude of mesencephalic morphine antinociception varied as a function of the

nociceptive test. On the tail-flick test, mesencephalic morphine antinociception was significantly and dose-dependently reduced by naltrindole doses of 0.5 (peak: 64%;

Figure 8. Alterations in latencies (A,C,E) and thresholds (B,D,F) in rats microinjected with vehicle (solid squares) or morphine (2.5 μg) in the PAG 24 h after pretreatment with vehicle (solid circles) or ß-FNA (5 μg, solid diamonds) microinjected into the RVM. The significantly increased latencies and thresholds across the time course following morphine were significantly reduced by ß-FNA (open stars). Significant reductions in peak (Panels C,D: 30 min) and total (Panels E,F) magnitudes of morphine antinociception were produced by a dose range (0.5-5 μg) of ß-FNA.

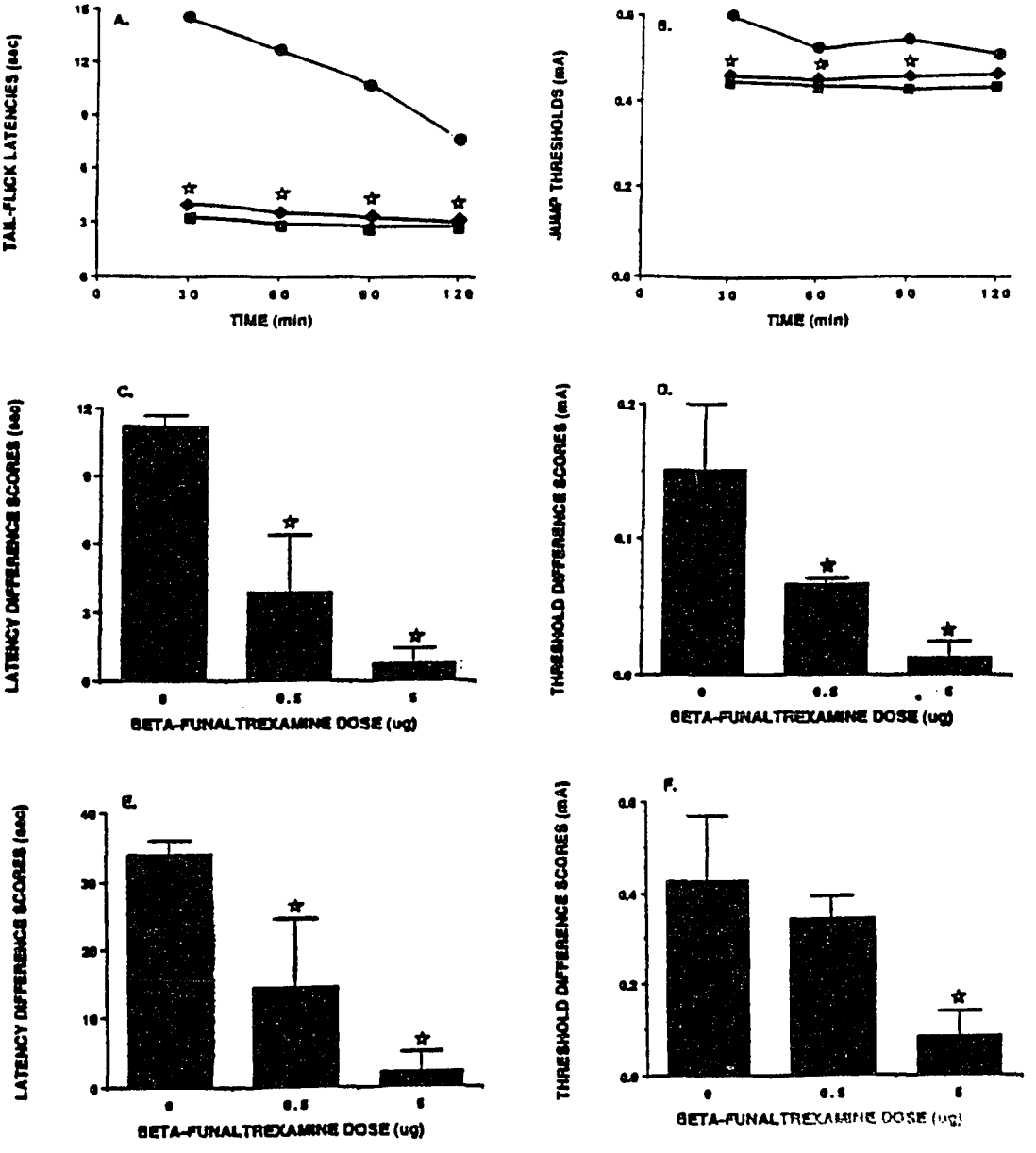
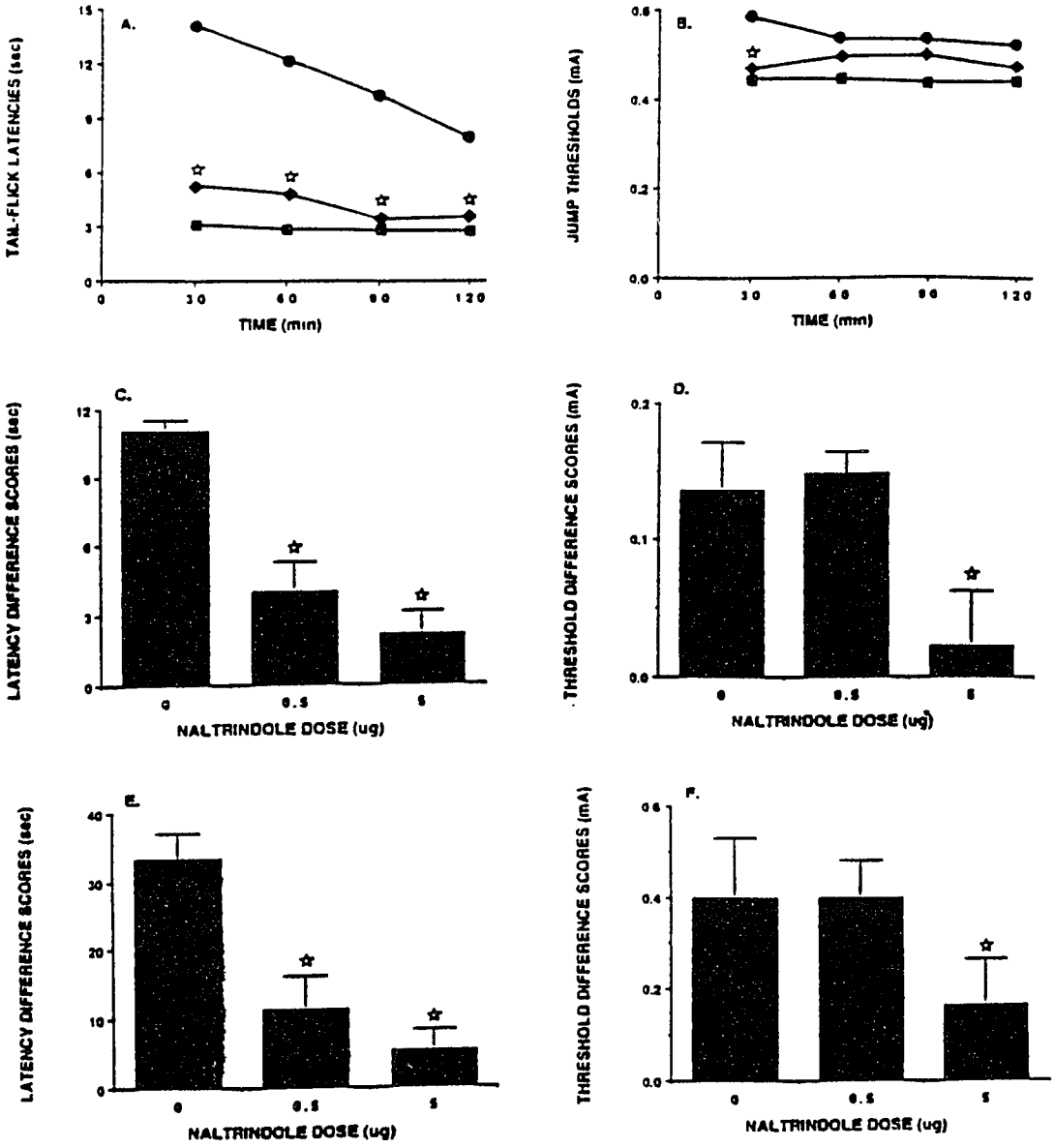

A.
TAIL-FLICK LATENCIES (sec)
TIME (min)
B.
JUMP THRESHOLDS (mA)
TIME (min)
C.
LATENCY DIFFERENCE SCORES (sec)
BETA-FUNALTREXAMINE DOSE (ug)
D.
THRESHOLD DIFFERENCE SCORES (mA)
BETA-FUNALTREXAMINE DOSE (ug)
E.
LATENCY DIFFERENCE SCORES (sec)
BETA-FUNALTREXAMINE DOSE (ug)
F.
THRESHOLD DIFFERENCE SCORES (mA)
BETA-FUNALTREXAMINE DOSE (ug)

Figure 9. Alterations in latencies (A,C,E) and thresholds (B,D,F) in rats microinjected with vehicle (solid squares) or morphine (2.5 μg) in the PAG 15 min after pretreatment with vehicle (solid circles) or naltrindole (5 μg, solid diamonds) microinjected into the RVM. The significantly increased latencies and thresholds across the time course following morphine were significantly reduced by naltrindole (open stars). Significant reductions in peak (Panels C,D: 30 min) and total (Panels E,F) magnitudes of morphine antinociception were produced by a dose range (0.5-5 μg) of naltrindole.


A.
TAIL-FLICK LATENCIES (sec)
TIME (min)
B.
JUMP THRESHOLDS (mA)
TIME (min)
C.
LATENCY DIFFERENCE SCORES (sec)
NALTRINDOLE DOSE (ug)
D.
THRESHOLD DIFFERENCE SCORES (mA)
NALTRINDOLE DOSE (ug)
E.
LATENCY DIFFERENCE SCORES (sec)
NALTRINDOLE DOSE (ug)
F.
THRESHOLD DIFFERENCE SCORES (mA)
NALTRINDOLE DOSE (ug)

total: 66%) and 5 (peak: 80%; total: 84%) μg in the RVM (Figures 9C and 9E). On the jump test, mesencephalic morphine antinociception was significantly reduced by only the naltrindole dose of 5 μg in the RVM (peak: 85%; total 59%) (Figures 9D and 9F).

Mesencephalic PAG antinociception and opioid antagonists in control placements: Significant differences were found among the different injection conditions (tail-flick: $F(4,31)= 39.46$, $p<.001$; jump: $F= 39.56$, $p<.001$), across the time course (tail-flick: $F(3,93)= 146.38$, $p<.001$; jump: $F= 241.46$, $p<.001$) and for the interaction between conditions and times (tail-flick: $F(12,93)= 12.88$, $p<.001$; jump: $F= 21.35$, $p<.001$). Morphine in the PAG significantly increased latencies across the time course and thresholds for up to 60 min when animals received vehicle in the control medullary placements (Table 5). Neither naltrexone nor naltrindole in the control medullary placements altered the magnitude of mesencephalic morphine antinociception on either the tail-flick or jump tests. While ß-FNA in the control medullary placements failed to alter mesencephalic morphine antinociception on the tail-flick test, it produced a slight and transient decrease in mesencephalic morphine antinociception on the jump test at 90 min following the injection.

Discussion

The present study found that administration of either the general opioid antagonist, naltrexone, the mu-selective

antagonist, ß-FNA or the delta-selective antagonist, naltrindole into the RVM significantly inhibited morphine antinociception elicited from the

TABLE 5. Alterations in mesencephalic morphine antinociception by pretreatment of either naltrexone, ß-FNA or naltrindole in medullary sites lateral and dorsal to the RVM.

CONDITION	POST-INJECTION TIME (min)			
	30	60	90	120
A. Tail-Flick Latencies (sec):				
Veh/Veh	2.47	2.39	2.32	2.28
Veh/Mor	11.14*	8.10*	4.35*	3.39*
Ntx/Mor	9.80*	7.40*	5.61*	3.80*
ß-FNA/Mor	10.06*	6.74*	5.31*	3.56*
Nti/Mor	10.36*	7.14*	6.07*	4.52*
B. Jump Thresholds (Ma):				
Veh/Veh	.444	.440	.449	.456
Veh/Mor	.768*	.631*	.510	.454
Ntx/Mor	.822*	.693*	.544*	.484
ß-FNA/Mor	.800*	.612*	.431+	.417
Nti/Mor	.797*	.693*	.549*	.417

*, significant difference relative to corresponding Veh/Veh (Dunnett comparison, $p<.05$); +, significant difference relative to corresponding Veh/Mor (Dunn comparison, $p<.05$). Veh = Vehicle; Mor = Morphine (2.5 µg); Ntx = Naltrexone (5 µg); ß-FNA = Beta-funaltrexamine (5 µg); Nti = Naltrindole (5 µg).

Note: The first treatment was administered into the RVM and the second treatment was administered into the PAG.

mesencephalic PAG. These effects were highly selective in that opioid antagonists microinjected into medullary cannula placements that were lateral and dorsal to the RVM failed to meaningfully alter mesencephalic morphine antinociception. This important control suggests that the inhibitory actions of naltrexone, ß-FNA and naltrindole upon mesencephalic morphine antinociception are acting at the RVM injection sites, and not non-specifically via diffusion. This is especially pertinent for ß-FNA in which a 24-h period elapsed between antagonist and agonist injections; the inability of ß-FNA to exert meaningful effects in control placements argues for its effectiveness in RVM sites. Thus, the RVM appears to be an effective placement through which both general and selective opioid antagonists can block opiate antinociception elicited from the PAG.

Naltrexone doses of 2.5, 5 and 10 μg in the RVM significantly inhibited the magnitude and duration of mesencephalic morphine antinociception on the tail-flick test. The two higher doses of naltrexone in the RVM significantly inhibited the magnitude and duration of mesencephalic morphine antinociception on the jump test as well. However, the two lower naltrexone doses (1 and 2.5 μg) in the RVM significantly enhanced mesencephalic morphine antinociception on the jump test, indicating test-specific antinociceptive effects. Whereas the tail-flick test is a spinally-mediated reflex (Grossman, Basbaum and Fields, 1982) present in spinalized

animals (Hayes et al., 1978), the jump test is dependent upon supraspinal and suprasegmental mechanisms for its integrity. Therefore, different populations of RVM neurons may be responsible for the modulation of the different nociceptive responses, and the lower naltrexone doses may be inadequate to block morphine's antinociceptive response when measured on the jump test. The mechanisms by which pretreatment of low naltrexone doses in the RVM enhances mesencephalic morphine antinociception are unknown, and must be subject to further study. One possible mediator may be differential opioid involvement in the activity of "on-cells" and "off-cells" in the RVM (see review: Fields et al., 1991). Also, the inability of naltrexone to completely block morphine's effects argues for the existence of two antinociceptive systems. Naltrexone has been found to produce "analgesia" (NIA) (Greeley, Le, Poulos and Cappell, 1988; Poulos, Knoke, Le and Cappell, 1990; Rochford and Stewart, 1987) and non-opioid stress-induced antinociception is potentiated by naltrexone (Kirchgessner, Bodnar and Pasternak, 1982; Yoburn, Truesdell, Kest, Inturrisi and Bodnar, 1987).

The present study also showed that administration of the mu antagonist, ß-FNA into the RVM dose dependently inhibited mesencephalic morphine antinociception on both nociceptive tests and produced the most pronounced effects upon mesencephalic morphine's peak actions. Finally, the present study showed that administration of the delta antagonist,

naltrindole into the RVM dose dependently inhibited mesencephalic morphine antinociception with more pronounced effects upon the tail-flick test than on the jump test. ß-FNA is quite potent in blocking mu receptors, while naltrindole blocks delta receptors but at about a 10-fold higher doses can also block mu receptors. This suggests that some of the effects of naltrindole might also be mu, not delta.

These data indicate that an opioid medullary synapse participates in the descending pain-inhibitory system projecting from the mesencephalic PAG through the RVM and terminating in the dorsal horn of the spinal cord (see reviews: Basbaum and Fields, 1984; Fields and Basbaum, 1978), and that mu and delta opioid receptor subtypes are each involved in this modulatory effect.

GENERAL DISCUSSION

The proposal that an endogenous supraspinal opioid pain-inhibitory system originated in the midbrain PAG, synapsed in the NRM, and projected to the substantia gelatinosa of the spinal cord through the dorsolateral funiculus (Basbaum and Fields, 1984; Fields and Basbaum, 1978) was generated by physiological and pharmacological data indicating that focal electrical stimulation or microinjection of morphine into either the PAG or the RVM produces antinociceptive effects (Malick and Goldstein, 1977; Pert and Yaksh, 1974; Tsuo and Jang, 1964; Yaksh et al., 1976; Yaksh and Rudy, 1978a; Takagi et al., 1977; Akaike et al., 1978; Azami et al., 1982; Dickenson et al., 1979; Levy and Proudfit, 1979; Besson et al., 1981; Vasko et al., 1984; Mayer and Price, 1976; Gebhart, 1982). The above manipulations also inhibit spinal dorsal horn nociceptive neurons (Guilbaud, Oliveras, Geisler and Besson, 1977; Gebhart, 1986; Bennett and Mayer, 1979; Clark, Edeson and Ryall, 1983). However, the precise neurotransmitter(s) in the RVM which mediate mesencephalic antinociception were not well delineated.

The goal of this dissertation was to clarify which receptors in the RVM serve to mediate opioid antinociception elicited from the midbrain PAG. Since the neurochemical neuroanatomy between the PAG and the medial medulla has been described as containing serotonin and enkephalins (e.g., Beitz, 1982a,b), and since both serotonergic (e.g., see

reviews: Peroutka, 1988a,b; Peroutka et al., 1990) and opioid (Lord et al., 1977; Martin et al., 1976; Pasternak and Wood, 1986) receptors have specific and distinct subtypes, this dissertation examined whether both general and specific serotonin and opioid antagonists administered into the RVM altered the antinociceptive response of morphine administered into the PAG. The effects of serotonergic antagonists and opioid antagonists administered into the RVM upon mesencephalic morphine antinociception will be discussed separately.

A. Serotonergic Antagonists in the RVM: The existence of serotonergic receptors on medial medullary neurons (Pazos et al., 1984, 1985; Pazos and Palacios, 1985), together with evidence that serotonergic mechanisms are involved in both opiate (Tennen, 1968; Yaksh, 1979) and stimulation-produced (Hayes, Newlon, Rosecrans and Mayer, 1977; Akil and Liebeskind, 1975) antinociception suggests that this neurotransmitter participates in descending pain inhibition. Further, the demonstration that methysergide microinjected into the NRM blocks the antinociception produced by electrical stimulation of the PAG (Aimone and Gebhart, 1986) indicates that serotonin serves as an important relay in the neurochemical connection between the PAG and the NRM. In the first experiment of this dissertation, the ability of methysergide microinjected into the RVM to significantly block the antinociception produced by microinjections of morphine

into the PAG further established the importance of this neurotransmitter in this supraspinal pathway.

With the discoveries of multiple receptor subtypes for certain neurotransmitters and the synthesis of agonists and antagonists with preferential affinities for specific receptor subtypes, there has been a concerted effort to determine the various functions of different receptor subtypes in certain behaviors. With regard to the serotonin receptor subtypes, investigations have been carried out to determine the differential roles the subtypes may play in male and female sexual behavior (Mendelson and Gorzalka, 1985a,b; 1986a,b), in ingestive behavior (Koch, Beczkowska, and Bodnar, 1992; Beczkowska et al., 1992a; Beczkowska and Bodnar, 1991a), and in different forms of environmental stress (Kiefel et al., 1989). Other research has focused on the role these subtypes play in the modulation of opioid antinociception. Systemic administration of the 5-HT_2 receptor antagonists ketanserin and pirenperone attenuates systemic morphine antinociception (Paul and Phillips, 1986; Paul et al., 1988). This effect appears to be mediated supraspinally since: a) pirenperone failed to affect morphine antinociception in rats with transected spinal cords (Paul and Pinel, 1990), and b) 5-HT_2 receptors have not been found in the spinal cord using either radioimmunoassay or autoradiographic techniques (Leysen et al., 1982; Monroe and Smith, 1983; Zemlan et al., 1990). Other research has demonstrated a role for both spinal and

supraspinal 5-HT_3 receptors in opioid antinociception (Ho and Takemori, 1990). Since both $5HT_2$ and $5HT_3$ receptor subtypes have been localized on medial medullary neurons (Pazos et al., 1985; Waeber et al., 1988), these serotonergic receptor subtypes appeared to be excellent candidates for the serotonergic participation in medullary modulation of mesencephalic morphine antinociception. The second experiment of this dissertation confirmed that microinjections into the RVM of either the specific 5-HT_2 receptor antagonist, ritanserin or the specific 5-HT_3 receptor antagonist, ICS 205930 significantly blocked the antinociception produced by microinjections of morphine into the PAG. These data provide substantial support for the importance of these receptor subtypes in the modulation of mesencephalic morphine antinociception.

The recent physiological characterization of two classes of modulatory neurons in the RVM which are involved in nociceptive processing has aided our understanding of nociceptive modulation in the RVM (see review: Fields et al., 1991). Whereas "on-cells" increase their firing rate just prior to the occurrence of a tail-flick response, "off-cells" pause in their firing prior to a tail-flick response. A role for serotonin receptors in the modulation of physiologically identified RVM cells is suggested by the ability of iontophoretically applied serotonin to produce excitation through a presumed 5-HT_2 mechanism and inhibition through a

presumed 5-HT_1 receptor mechanism (Davies, Wilkinson and Roberts, 1988a,b). Fields and co-workers (1991) have proposed that at least a subset of "off-cells" may contain serotonin, and that serotonin may be working as an "off-cell" transmitter. The results of the first two experiments suggest that mesencephalic morphine may activate antinociceptive circuits in the RVM in part through a serotonergic PAG-RVM pathway which utilizes 5-HT_2 and 5-HT_3 receptor subtypes.

Future Directions Concerning the Role of 5-HT Subtypes in Opioid Antinociception: This dissertation examined the PAG-RVM circuit as one of the components of the hypothesized model of descending pain-inhibitory pathways through which antinociception is elicited by microinjections of morphine into the PAG. As autoradiography confirmed 5-HT_2 and 5-HT_3 receptor subtypes on medial medullary neurons (Pazos et al., 1985; Waeber et al., 1988), the strategy taken in this dissertation for characterizing the nature of 5-HT receptors involved in the mediation of mesencephalic morphine antinociception was to directly administer 5-HT_2 and 5-HT_3 receptor antagonists into those brain areas (e.g., RVM) that are both rich in these receptor subtypes and are involved in supraspinal opioid antinociception. This strategy established that both 5-HT_2 and 5-HT_3 receptors in the RVM mediate mesencephalic morphine antinociception. Possible future directions for further investigating the interactions of serotonin receptor subtypes upon supraspinal opioid

antinociception might be to examine whether specific serotonin receptor subtypes located in the spinal cord (e.g., 5-HT_{1A}, 5-HT_{1C}, 5-HT_3) directly subserve mesencephalic morphine antinociception. Other studies should focus on the pharmacology underlying the RVM-spinal cord connection in terms of the specific serotonin subtypes. Indeed, the observations that electrical stimulation or microinjections of morphine into the PAG or the RVM produces an inhibition of spinal reflex activity (Azami et al., 1982; Dickenson et al., 1979; Kuraishi et al., 1979; Mayer and Liebeskind, 1974; Oliveras et al., 1975) and of dorsal horn nociceptive neurons (Guilbaud et al., 1977; Bennett and Mayer, 1979; Gebhart, 1986; Clark et al., 1983) supports the notion that supraspinal opioid antinociception involves descending connections. Previous research utilizing intrathecal injections of 5,6 dihydroxytryptamine, 5,7 diydroxytryptomine and general serotonin antagonists have found that these intrathecal manipulations block the antinociceptive effects of systemic morphine injections, microinjections of morphine into either the PAG or the RVM, and the antinociception elicited by electrical stimulation of either the PAG or the RVM (Tenen, 1968; Deakin and Dostrovsky, 1978; Kuraishi, Harada, Aratani, Satoh and Takagi, 1983; Yaksh, 1979; Yaksh and Wilson, 1979; Tseng and Tang, 1989; Hammond and Yaksh, 1984; Proudfit and Hammond, 1981; Jensen and Yaksh, 1986b; Hammond and Yaksh, 1984; Kuraishi et al., 1979; Barbaro et al., 1985; Satoh et

al., 1980). Thus, the line of research focusing on the specific serotonin receptor subtypes initiated in this dissertation should be used to examine PAG-spinal cord and RVM-spinal cord connections so as to precisely delineate which serotonin receptor subtypes modulate supraspinal opioid antinociception, and at what level of the PAG-RVM-spinal cord neuraxis.

Additionally, this dissertation utilized the general opioid agonist, morphine to elicit antinociceptive effects from the PAG. Future studies might examine whether pretreatment with either general or specific serotonin receptor antagonists in the RVM alters antinociception elicited by specific opioid receptor agonists administered into the PAG. This research should focus on mu-selective (DAMGO) and delta-selective (deltorphan) receptor subtype agonists, as well as beta-endorphin which are all involved in supraspinal opioid antinociception (Jensen and Yaksh, 1986a; Porreca et al., 1984; Bodnar et al., 1988; Fang et al., 1986, 1989; Mattia et al., 1991, 1992; Jiang et al., 1991a,b). Thus, these important neuroanatomical and neuropharmacological studies will help elucidate the role that serotonin plays in the mediation of supraspinal opioid antinociception.

B. Opioid Antagonists in the RVM: Much research has focused on the role that particular opioid receptor subtypes (mu, delta, kappa) play in the modulation of certain behaviors. Distinct opioid receptor subtypes have been shown

to have differential effects in ingestive behavior paradigms (Arjune and Bodnar, 1990; Arjune, Standifer, Pasternak and Bodnar, 1990; Arjune, Bowen and Bodnar, 1991; Beczkowska and Bodnar, 1991b; Beczkowska, Bowen and Bodnar, 1992b; Islam and Bodnar, 1990; Koch and Bodnar, 1993; Simone, Bodnar, Goldman and Pasternak, 1985). In such studies, the mu_1 antagonist, naloxonazine, modulates free, deprivation and stress-induced feeding, but fails to affect any other ingestive models. In contrast, the mu-selective antagonist, ß-FNA, blocks each of the above forms of feeding, but also modulates glucoprivic intake and deprivation-induced water intake. However, ß-FNA is relatively ineffective in altering palatable intake. The kappa antagonist, nor-binaltorphamine is effective in blocking palatable intake, but relatively ineffective in altering deprivation intake. Finally, delta antagonists, particularly naltrindole, only block intake of palatable solutions with no post-ingestive consequences. These data attest to the behavioral specificity of these selective antagonists.

The modulation of spinal and supraspinal opioid antinociception has been delineated with selective opioid receptor subtype agonists and antagonists. Antinociception following intrathecal administration of mu agonists is blocked by ß-FNA, but not naloxonazine, indicating a mu_2 mechanism of action (Paul et al., 1989). Delta and kappa agonists produce spinally-mediated antinociception which is blocked by their respective selective antagonists (Yaksh, 1984a,b; Porreca et

al., 1984, 1987; Wuster et al., 1980; Heyman et al., 1987, 1988). Opioid receptors, including both mu and delta subtypes have been localized supraspinally in the RVM (Atweh and Kuhar, 1977a,b; Goodman et al., 1980). As part of the intrinsic PAG-RVM connections (Abols and Basbaum, 1981; Beitz et al., 1983; Van Bockstaele et al., 1991; 1989), an enkephalin-immunoreactive pathway has been identified that projects from the PAG to the RVM (Beitz, 1982b). A second source of endogenous opioid input into the RVM is intrinsic opioid interneurons (Lewis et al., 1985). A role for the mu_1 receptor in supraspinal opioid antinociception is supported by naloxonazine's blockade of antinociception elicited by microinjections of morphine, DAMGO (mu) and DSLET (delta, mu_1) into the PAG, LC, NRM and NRGC (Bodnar et al., 1988). A supraspinal role for delta receptors (Jensen and Yaksh, 1986a; Porreca et al., 1984, 1987) is questioned by the failure of DPDPE, a $delta_1$-selective agonist, to produce antinociception following microinjection into the PAG, LC, NRM or NRGC (Bodnar et al., 1988). Kappa receptors do not appear to be participating in supraspinal antinociception (Friedman et al., 1981; Chavkin et al., 1982; Fang et al., 1989). The ability of EKC to produce antinociception following either intraventricular administration or concurrently into the PAG and LC is dependent upon mu_1 mechanisms (Bodnar et al., 1991). Thus, a number of studies (e.g., Bodnar et al., 1988; Fang et al., 1986; Smith, Perrotti, Crisp, Cabral, Long and Scalzitti,

1988) suggest a primary role for the mu receptor in supraspinal responses in these mesencephalic and medullary structures.

The last experiment of this dissertation found that administration of either the general opioid antagonist, naltrexone, the mu-selective antagonist, ß-FNA or the delta-selective antagonist, naltrindole into the RVM significantly inhibited morphine antinociception elicited from mesencephalic PAG. These effects were highly specific because opioid antagonists microinjected into medullary cannula placements that were lateral and dorsal to the RVM failed to meaningfully alter mesencephalic morphine antinociception. These data indicate that an opioid medullary synapse participates as part of the neurochemical makeup connecting the PAG to the RVM, and that both mu and delta opioid receptor subtypes are involved in this supraspinal pathway.

These data complement a number of recent physiological and pharmacological findings. In addition to the neurophysiological characterization of the functional links between the PAG and the RVM (see review: Fields et al., 1991), our laboratory (Rossi, Pasternak and Bodnar, 1993) has demonstrated multiplicative synergy between these structures. Sub-antinociceptive doses of morphine administered simultaneously into the PAG and into the RVM elicit a strong and prolonged antinociceptive response which shifts morphine's dose-response curve for each site significantly to the left.

Further, morphine antinociception elicited by simultaneous activation of the PAG and the RVM is blocked by naloxonazine, again implicating the mu receptor in this response.

That microinjections of either naltrexone or the mu-selective antagonist, ß-FNA into the RVM significantly inhibited mesencephalic morphine antinociception is in keeping with the foregoing data implicating the mu receptor in opioid supraspinal antinociception. However, the ability of microinjections of the delta-selective antagonist, naltrindole into the RVM to also significantly reduce mesencephalic morphine antinociception appears to implicate the delta receptor in the medullary opioidergic synapse mediating opioid supraspinal antinociception. If this is the case, one would expect that delta receptor agonists should elicit antinociception from these medullary sites. Recent work has indicated the existence of multiple delta receptors using in vitro (Negri et al., 1991) and in vivo techniques. Porreca and colleagues (Jiang et al., 1990a,b; 1991; Mattia et al., 1991, Mattia, Farmer, Takemori, Sultana, Portoghese, Mosberg, Bowen and Porreca, 1992) have indicated that DPDPE and [D-Ala^2, Leu^5, Cys^6]-enkephalin (DALCE) are respectively an agonist and antagonist at delta$_1$ sites, whereas deltorphan II and naltrindole are respectively an agonist and antagonist at delta$_2$ sites. The ability of such delta-selective ligands such as D-Ala^2-D-Leu^5-enkephalin (DADL) and DSLET to elicit antinociception when injected into the RVM (Bodnar et al.,

1988; Fang, Fields and Lee, 1986; Jensen and Yaksh, 1986b; Satoh et al., 1983), together with the inability of DPDPE (Bodnar et al., 1988) to do so have been explained by DADL and DSLET binding to the high-affinity mu_1 site (Hazum, Chang, Cuatrescasas and Pasternak, 1981; Itzhak and Pasternak, 1987) and the failure of DPDPE to bind to mu_1 sites (Clark, Itzhak, Hruby, Yamamura and Pasternak, 1986). Naltrindole's actions at $delta_2$ sites are quite specific since it inhibits the antinociceptive effects of ventricular DSLET and deltorphan, but not DPDPE. Conversely, DALCE inhibits the antinociceptive effects of DPDPE, but not DSLET or deltorphan (Jiang et al., 1990a, 1991; Sofuoglu et al., 1991). Similar effects have been observed in intrathecal studies (Mattia et al., 1992), and cross-tolerance fails to occur between different delta receptor subtype agonists (Mattia et al., 1991). Finally, our laboratory has preliminary data indicating that deltorphan produces antinociception following microinjections into the PAG and RVM, and that sub-antinociceptive doses of deltorphan into both sites displays synergy.

According to the physiological classification system of Fields and co-workers (see review: 1991), the RVM cells which increase their firing rate prior to the occurrence of the tail-flick response, "on-cells" are inhibited by systemic morphine or morphine microinjected into the PAG at doses sufficient to block the tail-flick response (Barbaro et al., 1986; Cheng et al., 1986), while "off-cells," which cease

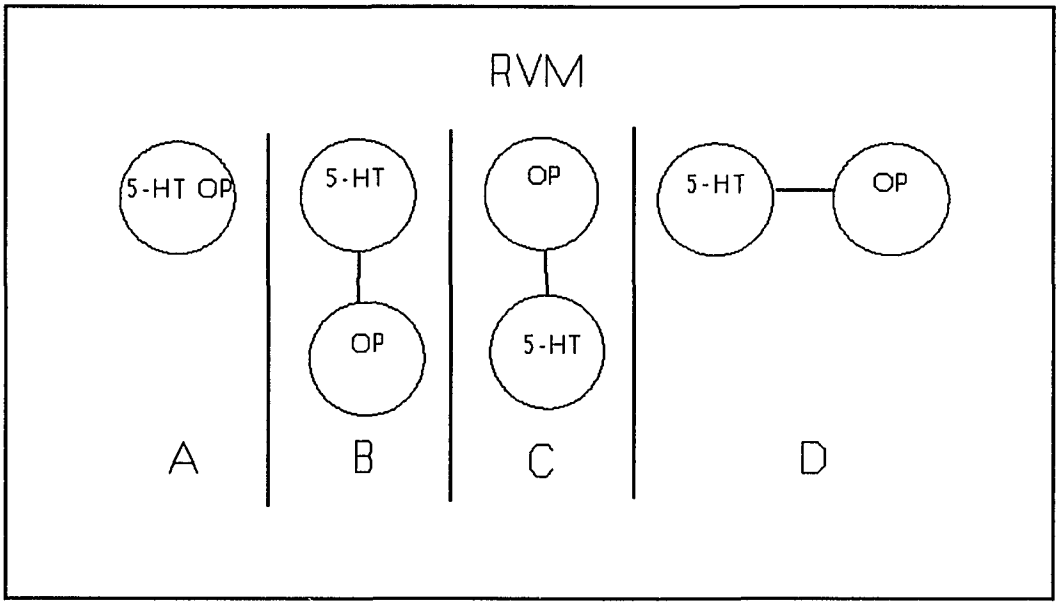
activity immediately prior to a tail-flick reflex are activated by systemic morphine (Fields et al., 1983) or microinjection of morphine into the PAG (Cheng et al., 1986). Further, glutamate-induced activation of medullary "on-cells" is inhibited by morphine pretreatment in the PAG, but not by intrathecal morphine pretreatment (Morgan, Heinricher and Fields, 1992). Finally, iontophoretic application of morphine directly upon medullary "on-cells" depressed their activity; this treatment failed to alter the activity of "off-cells" (Heinricher, Morgan and Fields, 1992). Thus, medullary "off-cells" appear not to be sensitive to direct application of opiates, and may be modulated by other transmitters. GABA has been proposed as a potential "off-cell" modulator in physiological studies (Heinricher, Haws and Fields, 1991). The ability of serotonin receptor antagonists in the RVM to reduce mesencephalic morphine antinociception in this dissertation and the ability of neurotensin antagonists in the RVM to enhance mesencephalic morphine antinociception (Urban and Smith, 1992) indicates that these other bioactive substances may be involved in the modulation of "off-cells" as well. In contrast, it appears that medullary "on-cells" may receive direct opioid input that modulates their responsiveness (Fields et al., 1991), and thus medullary "on-cells" may be the point of action at which opioid receptor subtype antagonists work to block mesencephalic morphine antinociception. Further autoradiographic and

neurophysiological studies are necessary to confirm such a relationship.

Future Directions Concerning the Role of Opioid Subtypes in Supraspinal Antinociception: Potential directions for this line of opioid research might be to administer specific mu-selective (DAMGO) and delta-selective (deltorphan) opioid receptor subtype agonists into the PAG following pretreatment in the RVM with general (naltrexone) or selective (B-FNA, naltrindole) opioid subtype antagonists. Given that analgesic synergy with morphine occurs between PAG-RVM, RVM-LC and PAG-LC placements (Rossi et al., 1993) and analgesic synergy with ethylketocyclazocine occurs between PAG-LC placements (Bodnar et al., 1991), these regional interactions suggest neurochemical links between these structures. There are enkephalinergic (Drolet, Van Bockstaele and Aston-Jones, 1992) and adrenergic (Pieribone and Aston-Jones, 1991) pathways between the NRGC and the LC, suggesting that morphine antinociception elicited from the RVM may be modulated through opioid and adrenergic synapses. Therefore, administration of general and specific opioid antagonists as well as adrenergic antagonists into the LC should be evaluated for its effects upon morphine antinociception elicited from either the RVM or the PAG.

In sum, this dissertation demonstrated that mesencephalic morphine administration activates antinociceptive circuits in the RVM. Although our knowledge of RVM circuitry is presently

unclear, several schematic models can be devised to aid in explaining the foregoing data. A 5-HT and an opioid receptor may coexist on the same RVM neuron (A). A 5-HT receptor may be in series with an opioid receptor (B), or vice versa; an opioid receptor may be in series with a 5-HT receptor (C). A 5-HT and an opioid receptor may be parallel to each other (D). With future advances made in elucidating the precise mechanisms of nociceptive processing in the RVM, a better understanding of the pharmacology underlying nociception will occur.



GENERAL CONCLUSIONS

The series of studies presented in this dissertation demonstrated that 5-HT_2 and 5-HT_3 receptor subtypes, as well as mu and delta opioid receptor subtypes participate in the modulation of supraspinal opioid antinociception by pathways projecting from the PAG to the RVM. Midbrain efferents to the RVM have been demonstrated to contain glutamate, neurotensin and substance P, in addition to serotonin and enkephalin (Beitz, 1982a,b). Recent work has implicated neurotensin neurons as being integral in the connection between the PAG and the RVM (Urban and Smith, 1992). Further, administration of nonselective excitatory amino acid receptor antagonists into the RVM significantly increased the electrical threshold for inhibiting the tail-flick response, implicating the importance of either glutamate and/or aspartate in the RVM in the mediation of electrical stimulation from the PAG (Aimone and Gebhart, 1986). Future research should examine these and other transmitter systems, in terms of PAG-RVM-spinal cord connections. This research should focus on specific receptor subtypes for the particular systems, when indicated.

Glossary

Beta-funaltrexamine (ß-FNA)	mu antagonist
[D-Ala^2,D-Leu^5]-Enkephalin (DADL)	delta agonist
[D-Ala^2,Leu^5,Cys^6]-Enkephalin (DALCE)	delta antagonist
[D-Ala^2,MePhe^4,Gly-ol^5]-Enkephalin (DAMGO)	mu agonist
[D-Ala^2,Glu^4]-deltorphan	$delta_2$ agonist
DLF	dorsolateral funiculus
[D-Pen$_2$,D-Pen^5]-Enkephalin (DPDPE)	delta agonist
[D-Ser^2,Leu^5]-Enkephalin (DSLET)	delta agonist mu_1 agonist
Ethyketocyclazocine (EKC)	kappa agonist
ICI 174864	delta antagonist
ICS 205930	5-HT_3 antagonist
LC	locus coeruleus
Morphine	mu agonist
Methysergide	general 5-HT antagonist
Naloxonazine	mu_1 antagonist
Naltrexone (NTX)	general opiate antagonist

Naltrindole (NTI)	$delta_2$ antagonist
NIA	naltrexone-induced analgesia
Nor-binaltorphimine (Nor-BNI)	kappa antagonist
NRGC	nucleus reticularis gigantocellularis
NRM	nucleus raphe magnus
NRPG	nucleus reticular paragigantocellularis
NTS	nucleus tractus solitarius
PAG	periaqueductal gray
Ritanserin	$5\text{-}HT_2$ antagonist
RVM	rostral ventral medulla
SIA	stress-induced antinociception
SPA	stimulation-produced antinociception
U50,488H	kappa agonist

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