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THE ROLE OF MEDULLARY CHOLINERGIC AND EXCITATORY AMINO ACID RECEPTORS MEDIATING MESENCEPHALIC OPIOID ANTINOCICEPTION

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

THE ROLE OF MEDULLARY CHOLINERGIC AND EXCITATORY AMINO ACID RECEPTORS MEDIATING MESENCEPHALIC OPIOID ANTINOCICEPTION

by

Marcello Spinella

Mentor: Dr. Richard J. Bodnar

Three experiments were performed to assess the respective roles of medullary cholinergic and excitatory amino acid receptors mediating mesencephalic opioid antinociception. Experiment 1 examined the effect of RVM excitatory amino acid (EAA) receptor antagonists on antinociception elicited by microinjection of morphine into the vlPAG. Mesencephalic morphine antinociception was eliminated by RVM administration of the competitive NMDA antagonist MK-801 on both the tail flick and jump tests, and was potently reduced by the non-competitive antagonist AP7. In contrast, RVM microinjection of the AMPA/kainate antagonist, CNQX failed to alter mesencephalic morphine antinociception on the tail-flick test, and produced only small, significant reductions in mesencephalic morphine antinociception on the jump test. Experiment 2 examined the effect of RVM cholinergic antagonists on antinociception elicited by microinjection of morphine into the vlPAG. The general muscarinic cholinergic antagonist, scopolamine, when microinjected into the RVM produced potent dose-dependent reductions in mesencephalic morphine antinociception on both the tail-flick and jump tests both in terms of the peak magnitude and the overall total duration of effects. The effects produced by M_1 and M_2 cholinergic receptor antagonists results were not as convincing, consistent, or specific as the

scopolamine effects, where both M_1 and M_2 receptors are blocked. The nicotinic cholinergic antagonist, mecamylamine, microinjected into the RVM significantly and dose-dependently reduced mesencephalic morphine antinociception on the tail-flick and jump tests. Experiment 3 evaluated the ability of EAA (MK-801and AP7) cholinergic antagonists (scopolamine, mecamylamine) to alter mesencephalic beta-endorphin (BEND) antinociception. These data are clearly dissociated from morphine antinociception elicited from the vlPAG following RVM pretreatment since neither NMDA nor cholinergic receptor subtype antagonists reduced BEND antinociception from the vlPAG. An opioid mechanism of BEND was confirmed by reversal of BEND antinociception from the vlPAG by pretreatment with naltrexone. The findings in this study showed anatomical specificity, since injections of antagonists outside the RVM were ineffective in reducing mesencephalic opioid Antinociception. Pharmacological specificity was evidenced by dose-response relationships and effects of various receptor subtype antagonists. Further, the RVM antagonists alone did not significanly alter basal nociceptive thresholds, ruling out a cancellation effect. Although BEND and morphine create antinociception through the μ receptor, it is not entirely surprising that the antinociception produced was differentially sensitive to EAA and cholinergic antagonists, since several pharmacological contrasts have been made between these two systems. To explain these differences, the differential sensitivity of BEND to splice variants of the μ receptor is hypothesized.

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Glossary

5-HT	serotonin
ACh	acetylcholine
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazole-proprionic acid
AP7	(-)-2-amino-7-phosphonoheptanoate
BEND	Beta-endorphin
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
DALCE	D-Ala^2, Leu^5, Cys^6-enkephalin
DAMGO	D-Ala^2, met-Phe^4, Gly(ol)^5-enkephalin
DPDPE	D-Pen^2, D-Pen^5-enkephalin
DLF	dorsolateral funiculus
EAA	excitatory amino acid
GABA	gamma-aminobutyric acid
LC	locus coeruleus
mGluR	metabotropic glutamate receptor
NE	norepinephrine
NMDA	N-methyl-D-aspartate
NRM	nucleus raphe magnus
NRGC	nucleus reticularis gigantocellularis
POMC	pro-opiomelanocortin
PMT	pontomesencephalotegmental complex
PPTN	pedunculopontine tegmental nucleus
RVM	rostroventromedial medulla
vlPAG	ventrolateral periaqueductal grey

I. INTRODUCTION

A. Anatomical Organization of Endogenous Supraspinal Pain Control

Endogenous control of pain is accomplished through a descending set of projections involving multiple structures from supraspinal to spinal levels, and combined with findings from pharmacological and electrophysiological studies, an approximate working model of antinociception has been created (Basbaum and Fields, 1984).

1. Mesencephalon: The periaqueductal grey (PAG) is an area of cells surrounding the mesencephalic cerebral aqueduct (Bandler and Shipley, 1994). It is a region of small, densely-packed cells which are difficult to divide up morphologically (Gioia, Bianchi and Tredici, 1984). However, anatomical tracing studies have shown that regions of the vlPAG project as distinct functional units (Beitz, Shepard and Wells, 1983; Van Bockstaele, Aston-Jones, Pieribone, Ennis and Shipley, 1991). It receives sensory input, and integrates analgesic, defensive/aggressive, and autonomic behaviors (Mayer and Price, 1976; Bandler and Shipley, 1994). The vlPAG connects reciprocally to several areas of the brain, receiving afferents from numerous forebrain sites including frontal neocortex, insular cortex, infralimbic and prelimbic cortex, anterior cingulate cortex, medial precentral cortex, central nucleus of the amygdala, and the medial preoptic hypothalamus (Van Bockstaele et al., 1991; Bandler and Shipley, 1994). Further connections were reported with brainstem regions including the superior colliculus, cuneiform nucleus, NRM, NRGC, NRPG, nucleus of the solitary tract (NTS) and nucleus ambiguus (Mitchell, Dean and Redgrave, 1988; Redgrave, Dean, Mitchell, Odekunle, 1988; Beitz, 1982; Bandler and Tork, 1987; Van Bockstaele, Pieribone and Aston-Jones,

1989). Conversely, the vlPAG receives projections from several brainstem nuclei including the nucleus cuneiformis, the pontine reticular formation, and the LC. The pontine reticular formation and the LC have been proposed to regulate the nociceptive afferents which activate neurons in the vlPAG (Beitz et al., 1983; Shipley, McLean and Behbehani, 1987; Smith, Perotti, Crisp, Cabral, Long and Scaizitti, 1988).

Antinociception is produced by either electrical or neurochemical opioid stimulation of the vlPAG (see reviews: Bausbaum and Fields, 1984; Akil, 1984; Proudfit, 1988). The vlPAG and dorsal raphe nucleus, contain pro-opiomelanocortin (POMC)-positive terminals (Khachaturian et al., 1982). Enkephalinergic cells, by comparison, are found in the caudal and ventrolateral region of the vlPAG, and shift to more dorsal regions as one proceeds rostrally (Beitz, 1982). In addition, dynorphin cells are found ventral to the aqueduct, along the rostral-caudal extent of the vlPAG (Burnett and Gebhart, 1991).

2. Metencephalon: The LC is a predominant source of noradrenergic projections in the central nervous system, with extensive forebrain, brainstem and spinal projections (Lindvall and Bjorklund, 1974). Particularly relevant to antinociception are projections from the LC to the NRM and NRGC of the RVM (see review: Moore and Bloom, 1979). Stimulation of the LC faciltates antinociception produced by both electrical stimulation (Segal and Sandberg, 1977) and morphine microinjection (Bodnar et al., 1988,1991). The LC receives reciprocal inputs from the dorsal medulla and the NRGC (Aston-Jones, Ennis, Pieribone, Nickell and Shipley, 1986; Ennis and Aston-Jones, 1986, 1987; Ennis, Shipley, Behbani, Van Bockstaele and Aston-Jones, 1991). It has been suggested that

ceruleospinal projections mediating antinociception are activated by projections from the RVM neurons (Clark and Proudfit,1991). Antinociception elicited by the vlPAG and LC depends in part on alpha-2 receptors in the dorsal horn (Peng , Lin, and Willis, 1996). Antinociception elicited by stimulation of the vlPAG was attenuated by an alpha-2 agonist (clonidine), and facilitated by the alpha-2 adrenoceptor antagonists idazoxan and yohimbine (Peng , Lin, and Willis, 1996).

Another metencephalic area that is part of the pontine reticular formation, the pedunculopontine tegmental nucleus (PPTN), is a major brainstem source of cholinergic cells, which mediate various functions, including antinociception. The PPTN receives projections from the cerebral cortex, pallidum, and substantia nigra. Ascending PPTN projections travel to the midbrain, diencephalon, basal forebrain, and cerebral cortex. Descending PPTN projections extend to the medullary reticular formation and spinal cord (Paxinos, 1990). Relevant to antinociceptive circuits, histological investigations have shown projections from the PPTN to the NRM (Iwamoto and Marion, 1993). Another source of cholinergic fibers from the pontomesencephalotegmental (PMT: Woolf and Butcher, 1986) complex projects to the dorsal and median raphe nuclei, the NRM and medullary reticular nuclei (Woolf and Butcher, 1989; Mitani et al., 1988; Rye et al., 1988; Shiromani et al., 1988). Finally, the RVM also receives cholinergic innervation from the paragigantocellular nucleus of the ventral medulla (Sherriff and Henderson, 1994). The cholinergic fibers, in turn, project from the RVM to the spinal cord (Bowker and Westlund, 1983; Jones et al., 1986).

3. Myelencephalon: A critical brainstem area in antinociception is the RVM.

Zagon (1993) organized the RVM into three distinct subregions: the midline raphé region, the lateral paragigantocellular-gigantocellular region and the rostro-ventrolateral reticular nucleus. Opioid antinociception in the RVM is mediated by two sites: a) the paramedial medulla which includes the NRGC, and b) the medial medulla which includes the NRM and NRGC pars alpha (Jensen and Yaksh, 1986). Other structures in the RVM are the nucleus reticularis magnocellularis, the nucleus paragigantocellularis and the lateral reticular nucleus (Fields et al., 1991).

The NRGC receives afferent input from the ascending anterolateral spinal system, and its neurons are excited by noxious stimuli. Zagon (1995) has identified a complex reciprocal circuit of connections within the RVM. The RVM serves as an integration site for many autonomic and visceral processes including antinociception. There are inputs from the raphe pallidus and raphe obscurus to the RVM. Whereas the raphe pallidus plays a role in descending spinal control, the raphe obscurus serves in a coordinating role over internal RVM activity. Projections from the NRM appear to be limited to within the RVM, whereas the gigantocellular nuclei have substantial spinal projections (Zagon, 1995).

Projections from the vlPAG to the RVM, and then from the RVM to the dorsal horn of the spinal cord have been identified using anatomical and physiological approaches (Fields et al., 1991; Yeung and Rudy, 1980a; Yaksh, 1979; Proudfit and Anderson, 1975; Miyamoto, Morita, Kitabata, Yamanishi, Kishioka, Ozaki and Yamamoto, 1991). This pathway utilizes several neurotransmitters found in the RVM, including 5-HT (Clements et al., 1985), norepinephrine (NE) (Moore and Bloom, 1979),

GABA (Clements et al., 1987), enkephalin, neurotensin (Beitz, 1982), and excitatory amino acids. Serotonergic neurons of the NRM include two populations with colocalized neurotransmitters: a) neurons releasing 5-HT, Substance P, and thyrotropin releasing hormone (TRH), and b) neurons releasing 5-HT and enkephalin (Hokfelt et al., 1976).

B. Opioid Neuropharmacology of Supraspional Pain Control

1. Endogenous Opioid Peptide Families. Although opiate alkaloids have been used in the control of pain throughout human history, a firm understanding of endogenous central inhibition of pain has occurred fairly recently. A significant advance in this respect was made by Hughes and colleagues who isolated two opioid pentapeptides, methionine-enkephalin (H-Tyr-Gly-Gly-Phe-Met-OH) and leucine-enkephalin (H-Tyr-Gly-Gly-Phe-Leu-OH) from bovine and porcine brain (Hughes, 1975a). Both met- and leu-enkephalin were found to be potent agonists at opioid receptors as demonstrated by the dose-related inhibition of contractions in the guinea pig ileum and mouse vas deferens bioassays (Creese and Snyder, 1975; Hughes, Kosterlitz and Leslie, 1975).

Although such opioid peptides were identified to possess a common amino acid core, Tyr-Gly-Gly-Phe, they may be derived from different prohormone precursors which have distinct genetic coding and neuroanatomical distribution (Burckhardt, Frederickson and Pasternak, 1982; Eipper, 1980; Guillemin et al., 1976; Kimura, Lewis, Stern, Rossier, Stein and Undenfriend, 1980; Herz, 1987; Basbaum and Fields, 1984; Akil, Watson, Young, Lewis, Khachaturian and Walker, 1984). Classic endogenous

opioid peptides are derived from one of three families of precursors: a. pro-opiomelanocortin (POMC), b. pro-dynorphin, and c. pro-enkephalin.

a. POMC Opioids: POMC contains the peptide beta-lipotropin (B-LPH), a 91 amino acid peptide, contains the opioid peptide BEND as its C-terminal fragment (61-91) (Eipper and Mains, 1978; Mains, Eipper and Ling, 1977). BEND was shown to have opioid activity in bioassays (Cox et al., 1975). Two other opioid peptides were isolated from the hypothalamus and pituitary which were termed alpha-endorphin (B-LPH: 61-76) and gamma-endorphin (B-LPH: 61-77) (Guillemin, Ling and Burgus, 1976). The two major nuclei which produce BEND in the brain are in the arcuate and periarcuate nuclei of the hypothalamus, and in the dorsal caudal medulla in the nucleus of the solitary tract (Khachaturian, Lewis, Schaefer and Watson, 1985; Watson, Akil, Richard and Barchas, 1978; Bloom, Rossier, Battenberg, Bayon, French, Henricksen, Siggins, Segal, Browne, Ling and Guillemin, 1978). Arcuate neurons producing POMC have rostral projections to areas of the basal forebrain, including the preoptic hypothalamus, amygdala, septal nuclei, and bed nucleus of the stria terminalis. Caudal projections reach the periventricular thalamus, vlPAG, NRM, NRGC, NTS, lateral reticular nuclei, parabrachial nucleus, nucleus ambiguus, and the dorsal motor nucleus of the vagus (Guillemin, Ling and Burgus, 1976; Khachaturian et al., 1985; Watson and Mayer, 1982).

b. Proenkephalins: The proenkephalin precursor is cleaved into several opioid peptides which include leu-enkephalin, met-enkephalin, met-enkephalin-Arg-Phe (MERF), and met-enkephalin-Arg-Gly-Leu (MERGL) (Kimura, Lewis, Stern, Rossier, Stein and Udenfriend 1980; Comb, Herbert and Crea, 1982). Enkephalinergic neurons

are distributed, primarily as interneurons, throughout several levels of the neuraxis including the telencephalon (cerebral cortex, olfactory tubercle, amygdala, hippocampus, bed nucleus of the stria terminalis), diencephalon (hypothalamus, periventricular, and lateral geniculate thalamus), mesencephalon (PAG, corpora quadrigemina and interpeduncular nucleus), met-/myelencephalon (NRM, NRGC, NTS, nucleus reticularis paragigantocellularis, lateral reticular nucleus and spinal trigeminal nucleus), and the dorsal horn of the spinal cord) (Hokfelt, Elde, Johansson, Terenius and Stein, 1977; Khachaturian, Lewis, Hollt and Watson, 1983; Sar, Stumpf, Miller, Chang and Cuatrecasas, 1978).

c. Pro-dynorphin. Pro-dynorphin is cleaved into three opioid peptides: alpha and beta-neoendorphin, dynorphin A and dynorphin B (Goldstein, Fischli, Lowney, Hunkapiller and Hood, 1981; Kangawa, Minamino, Chino, Sakakibara and Matsuo, 1981). Dynorphin A may be further cleaved into such potent fragments as dynorphin A(1-17) and dynorphin A(1-8) (Goldstein et al., 1981; Seizinger, Hollt and Herz, 1981; Suda, Tozawa, Tachibana, Demura and Shizume, 1982). Pro-dynorphin neurons are also distributed in several levels of the neuraxis including telencephalon (cerebral cortex, hippocampus, striatum and amygdala), diencephalon (suprachiasmatic, supraoptic, paraventricular, and arcuate hypothalamus), mesencephalon (PAG), met-/myelencephalon (NTS, lateral reticular nucleus and spinal trigeminal nucleus), and the spinal dorsal horn (Khachaturian et al., 1985).

2. Opioid Receptor Subtypes. An opioid receptor was first proposed in 1954, but it wasn□t until 1973 that researchers in three laboratories independently demonstrated

their existence (Beckett and Casy, 1954, 1956; Portoghese 1965, 1966; Pert and Snyder, 1973; Simon, Hiller, and Edelman, 1973; Terenius, 1973). Based on data from cross-tolerance studies, Martin and colleagues (1976) proposed the existence of three subtypes of opioid receptors, and named them after the agonist used to characterize it: μ (morphine), κ (ketocyclazocine), and sigma (SKF-10,047). Subsequently the sigma receptor was recognized not to be an opioid receptor since it does not bind the general opioid antagonist naloxone (Zukin, Brady, Slifer and Balster, 1984; Vaupel, 1983). Lord et al. (1977) proposed the existence of a δ receptor (named after the mouse vas deferens bioassay) based upon data from bioassay and binding studies which yielded differences between the effects of morphine and enkephalin. The different opioid receptors have themselves been divided into subtypes, which are differentially distributed throughout the nervous system.

a. Mu Receptors. μ receptors are widely distributed throughout the brain. Highest densities of receptors are found in the neocortex, striatum, nucleus accumbens, thalamus, hippocampus, amygdala, tectum, NTS, spinal trigeminal nucleus, and dorsal horn of the spinal cord. Moderate μ binding has been observed in the vlPAG, and raphe nuclei. Little binding is found in the hypothalamus and globus pallidus (Mansour, Khachaturian, Lewis, Akil and Watson, 1988).

μ receptors are further divided into μ_1 and $\mu_2$subtypes based on pharmacological assays, biochemistry, and computer modeling (see review Pasternak and Wood, 1986). Both μ subtypes have a high affinity for morphine, but the μ_1 site binds morphine more potently, and has different selectivities for other opioids (Pasternak and Wood, 1986).

Thus, the μ_1 site binds morphine, ethylketocyclazocine, enkephalin, and BEND with equal affinity. μ_2 receptors also bind morphine with high affinity but have low affinity for other opioids. The μ subtypes are distinguished from one another by use of selective antagonists. Beta-funaltrexamine (B-FNA) irreversibly antagonizes both μ receptor subtypes equally well, but naloxazone and naloxonazine selectively antagonize the μ_1 but not the $\mu_2$site (Ward et al., 1982; Hahn et al., 1982). D-Ala^2, met-Phe^4, Gly(ol)^5-enkephalin (DAMGO) has been developed as a selective μ agonist, and B-FNA has been developed as a selective μ antagonist (Handa, Lane, Lord, Morgan, Rance and Smith, 1981; Portoghese, Larson, Sayre, Fries and Takemori, 1980; Takemori, Larson and Portoghese, 1981).

Recent techniques in the field of molecular biology have been employed to the study of opioid receptors, corroborating findings obtained from pharmacological bindng studies as well as providing novel insights. The cloned μ opioid receptor (MOR1) (see reviews: Uhl and Pasternak, 1994) has been probed using antisense oligodeoxynucleotides (AS ODN) which are believed to block their phenotypic expression. AS ODN directed at different exons have indicated the existence of multiple μ receptors: μ_2 and a novel receptor for morphine-6ß-glucuronide (M6G) (see review: Pasternak and Standifer, 1995). Whereas ODNs directed at exons 1 and 4 (but not 2 or 3) of the MOR1 clone block μ-induced antinociception. ODNs directed at exon 2 and 3 (but not 1 or 4) block M6G-induced antinociception (Pasternak and Standifer, 1995).

b. Delta Receptors. Localization of δ receptors is densest in the olfactory bulbs, neocortex, striatum, nucleus accumbens, and amygdala. Little binding has been observed

in the thalamus, hypothalamus, and brainstem (Mansour et al., 1988). Pharmacological characterization of δ receptors has utilized the δ -selective agonists D-Ser^2, Leu^5-enkephalin, Thr^6 (DSLET) and D-Ala^2, D-Leu^5-enkephalin (DADLE) (Lord, et al., 1977; Mosberg, Hurst, Hruby, Gee, Yamamura, Galligan and Burks, 1983a). Naltrindole and ICI 174864 have served as a general δ antagonist (Cotton, Giles, Miller, Shaw and Timms, 1984), although more selective antagonists have revealed the existence of δ_1 and δ_2 subtypes (Negri, Potenza, Corsi and Melchiri, 1991). The δ_1 receptor has been characterized using the agonist D-Pen^2, D-Pen^5-enkephalin (DPDPE) and the antagonist D-Ala^2, Leu^5, Cys^6-enkephalin (DALCE) (Mosberg, Hurst, Gee, Yamamura, Galligan and Burks, 1983b; Bowen, Hellewell, Kelemen, Huey and Steward, 1987; Jiang, Bowen, Mosberg, Rothman and Porreca, 1990). The δ_2 receptor has been characterized using the agonist D-Ala^2-deltorphan II (Jiang, Heyman, Sheldon, Koslo and Porreca, 1990) and the antagonist naltrindole isothiocyanate (Portoghese, Sultana, Nagase and Takemori, 1988a; Portoghese, Sultana and Takemori, 1988b; Sofuoglu, Portoghese and Takemori, 1991). Further, the δ_1 and δ_2 receptors have been dissociated in behavioral assays (Mattia, Vanderah, Mosberg and Porreca, 1991; Jiang, Takemori, Sultana, Portoghese, Bowen, Mosberg and Porreca, 1991).

The δ opioid receptor was the first opioid receptor to be cloned (Evans, Keith, Morrison, Magendzo and Edwards, 1992; Keiffer, Befort, Gaveriaux-Ruff and Hirth, 1992). This allowed for development of AS ODN which presumably block δ receptors. In support of this, AS ODN developed with the cloned DOR1 (δ opioid receptor) have been demonstrated to decrease δ receptor binding by 40-50% (expressed in NG108-15

cells) and reduce mRNA levels by 25% (Standifer, Chien, Wahlestedt, Brown and Pasternak, 1994). However, this down-regulation is not associated with associated with altered DOR mRNA levels (Lee et al., 1997).

c. Kappa Receptors. Densest κ receptor binding has been observed in the striatum, nucleus accumbens, amygdala, hypothalamus, neurohypophysis, median eminence, and NTS. Moderate levels of binding have been observed in the vlPAG, raphe nuclei, spinal trigeminal nucleus, and the dorsal horn of the spinal cord (Mansour et al., 1988). U50,488H has been used extensively as the prototypical κ agonist (VanVoightlander, Lahti and Ludens, 1983) and nor-binaltorphimine (NOR-BNI) has been employed as a selective antagonist (Portoghese, Lipkowski and Takemori, 1987; Takemori, Ho, Naeseth and Portoghese, 1988). The κ receptors have been further subdivided into κ_1, κ_2 and κ_3 subtypes (Zukin, Eghbali, Olive, Unterwald and Tempel, 1988; Rothman, Bykov, deCosta, Jacobson, Rice and Brady, 1990). These subdivisions are based on data which suggest that U50,488H and NOR-BNI are κ_1 ligands, and naloxone benzoylhydrazone (NalBzoH) is a selective κ_3 ligand (Clark, Liu, Price, Hersh, Edelson and Pasternak, 1989; Gistrak, Paul, Hahn and Pasternak, 1989; Paul, Levinson, Howard, Pack, Hahn and Pasternak, 1990).

The κ_1 and κ_3 receptors have been cloned (KOR1 and KOR3, respectively). ODNs directed at the KOR3 receptor have been shown to block the analgesic actions of NalBzoH, but not those produced by ligands for the μ, δ, or κ_1 receptors (Pan , et al., 1995; Pan, Cheng, Xu and Pasternak, 1994; Uhl, Childers and Pasternak, 1994).

C. Neurophysiology and Neurochemistry of Antinociceptive Circuits

There are three functional classes of neurons indentified in the RVM which participate in antinociception based on the correlation of their activity with nocefensive reflexes: "on-cells," "off-cells," and "neutral cells" (Fields , Barbaro and Heinricher, 1988; Fields et al., 1991). "On-cells" and "off-cells" are activated by electrical stimulation of the vlPAG, and they are mutually inhibitory. "On-cells" may actually have a facilitatory role in nociception. They show an increase in firing before a nocifensive response, such as the tail-flick to activate the withdrawal reflex (Vanegas, Barbaro and Fields, 1984). Neutral cells, in contrast, do not show any change in firing related to execution of the jump test (Fields et al., 1983). Administration of morphine suppresses the firing of "on-cells" (Mason and Fields, 1989), and reversal of morphine-induced antinociception is associated with a high rate of RVM "on-cell" firing (Bederson, 1990).

"Off-cells," in contrast, have an inhibitory effect on nociceptive transmission, and pause in firing 400 msec before nocifensive responses, allowing nociceptive transmission and execution of the nocifensive response. Administration of morphine either systemically (Fields et al., 1983b) or by microinjection into the vlPAG (Cheng et al., 1986) in doses sufficient to suppress the tail-flick causes continuous firing of the "off-cells."

Because the direct cellular actions of opioid receptor agonists are generally regarded to be inhibitory, opioid excitation of"off-cells" is likely due to disinhibition (Fields et al, 1983). Consistent with this is the observation that enkephalin-immunoreactive axonal swelling are apposed to intracellularly-labeled RVM "on-cells"

(Back et al. 1990). Since GABA mediates the RVM "off-cell" pause, it is suggested that a subset of RVM "on-cells" are GABAergic inhibitory interneurons (Pan and Williams, 1990). One putative "off-cell" neurotransmitter is 5-HT, and microinjection of 5-HT into the RVM has an antinociceptive action (Aimone and Gebhart, 1986) Systemic morphine increases the concentration of 5-HT metabolites in the RVM (Rivot, 1989). Also, depletion of spinal cord 5-HT by the neurotoxin 5,7-dihydroxytryptamine blocks the antinociceptive effect of morphine microinjected into the RVM (Vasko et al, 1984). Given the fact that the "off-cells" are the only RVM neurons activated by opioid administration, and considering the above-mentioned observations about serotonin function, it appears that at least part of the "off-cells" contain 5-HT. Significant extrinsic sources of 5-HT to the RVM are neurons in the vlPAG and the midbrain B8 and B9 cell groups (Beitz et al., 1983). Peroutka (1988) and colleagues identified at least 4 subtypes of serotonin receptors in pain-inhibitory circuits, including 5-HT_1, 5-HT_2, and 5-HT_3. However, spinal administration of 5-HT_1 agonists actually produce hyperalgesia (Millan and Coelpaert, 1991a,b). Adminstration of a general 5-HT antagonist, methysergide, into the NRM/NRGC reduced morphine antinociception elicited by morphine in the vlPAG, suggesting a serotonin synapse in this pathway (Kiefel et al., 1992). In addition to serotonin, the pathway from the vlPAG area to the RVM also contains EAA, Neurotensin, Somatostatin, Substance P and Norepinephrine (Wiklund et al., 1988; Beitz, 1983, Takagi et al., 1981). Neurotensin also has a modulator role and, when microinjected into the RVM, produces a dose-related suppression of tail-flick latencies (Fang et al, 1987) and alters morphine antinociception (Urban an Smith 1993, 1994).

Norepinephrine through alpha1 and alpha2 receptors exerts respective facilitatory and inhibitory effects on "on-cells"and therefore respectively decrease and increase jump test latencies (Henricher, MM and Haws, CM, 1992). Noradrenergic input to the RVM derive, in part, from the A5 and A7 cell groups in the pons, as well as from the A1 catecholamine cell group in the ventrolateral medulla (Kwiat and Basbaum, 1992; Dong and Shen, 1986).

D. Exogenous Elicitation of Antinociception

1. Differences between morphine and BEND antinociception. Although BEND antinociception (e.g. Loh, Tseng, Wei, and Li, 1976; Tseng, Oswald, Loh, and Li, 1979) was originally thought to be mediated through the μ receptor (see review: Akil, Watson, Young, Lewis, Khachaturian, and Walker, 1984), subsequent pharmacological and physiological studies clearly dissociated antinociceptive responses elicited by ventricular morphine and BEND. First, intrathecal naloxone blocks ventricular BEND, but not morphine antinociception (Tseng and Fujimoto, 1985). Immunoreactive spinal met-enkephalin is released following BEND, but not morphine following their injection into either the lateral ventricles, posterior nucleus accumbens, medial preoptic area or arcuate nucleus of the hypothalamus (Tseng, Higgins, Hong, Hudson, and Fujimoto, 1985; Tseng and Wang, 1992). Antibodies raised against met-enkephalin blocks ventricular BEND, but not morphine antinociception (Tseng and Suh, 1989). Spinal δ, but not μ opioid receptors appear to modulate ventricular BEND antinociception (Suh and Tseng,1990a). Further, ventricular morphine and BEND fail to develop analgesic cross-tolerance (Suh

and Tseng,1990c) and are differentially altered by pentobarbital anesthesia Tseng and Tang, 1990). Spinal cholecystokinin systems may modulate antinociception induced by ventricular BEND as compared to ventricular morphine (Suh and Tseng,1990b; Suh and Tseng, 1992; Tseng and Collins, 1992). Morphine and BEND only display additive antinociception following ventricular and intrathecal administration (Roerig, Fujimoto, and Tseng, 1988).

Differences between morphine and BEND antinociception also occur in the vlPAG. Barbiturate anesthesia reduces morphine antinociception, but enhances BEND antinociception (Smith, Robertson, and Monroe, 1992). Although antinociception induced by either morphine or BEND in the vlPAG was blocked by either general or μ-selective antagonists (e.g., CTOP: [Smith, Robertson, Monroe, Leedham, and Cabral, 1992]), BEND antinociception is differentially sensitive to these antagonists when compared with morphine (Hawranko, Monroe, and Smith, 1994; Monroe, Hawranko, Smith and Smith, 1996), suggesting the involvement of separate opioid receptors. Finally, whereas BEND antinociception in the vlPAG is dependent upon a spinal opioid component, morphine antinociception in the vlPAG is dependent upon spinal noradrenergic and serotonergic components (Suh, Fujimoto, and Tseng, 1989; Suh, Tseng, and Li, 1988; Tseng and Collins, 1991; Tseng and Tang, 1990).

2. Evaluative Techniques Examining Supraspinal Opioid Antinociception. Two functional techniques have been applied to determine the circuitry underlying supraspinal opioid antinociception: a.) opioid synergy studies, and b.) RVM antagonists effects upon morphine antinociception from the vlPAG.

a. Opioid Synergy. Several studies have found synergistic actions of opioids. Synergy is operationally defined as a greater than additive effect when subthreshold doses of an agonist are applied. Yeung and Rudy (1980) found that concurrent i.t. and i.c.v. injection of morphine interacted in a supra-additive manner to induce antinociception, and that the strength of this mutual potentiation was apparently greatest at a high ratio of supraspinal to spinal dosage. At low to moderate systemic morphine doses, both the spinal and supraspinal substrates have an obligatory involvement in mediating the analgetic effect, whereas at high systemic doses, spinal and supraspinal agonist actions become capable of mediating antinociception by independent actions.

A series of studies by Bodnar and colleagues (Bodnar at al., 1991; Rossi et al., 1993, 1994) have shown that subthreshold doses of morphine which are inactive when administered alone, will produce potent antinociception when simultaneously coadministered into pairs of the following three regions: vlPAG and RVM, vlPAG and LC, and RVM and LC (Rossi et al., 1993). The most sensitive interactions occured for subthreshold morphine doses in the vlPAG and RVM, which was completely blocked by the $\mu_1$antagonist, naloxonazine. A second study Bodnar et al., 1991) found that microinjection of the putative κ_1 agonist ethylketocyclazocine, or U50488H into either the vlPAG or LC alone failed to produce antinociception. However, co-administration of ethylketocyclazocine, but not U50488H into the vlPAG and LC produced a robust, naloxonazine-sensitive antinociception, implying actions of μ_1 receptors in this response. This particular study implicated the importance of regional analgesic interactions, as opposed to condition in which only one microinjection site is utilized. It was also shown

that coadministration of a low-analgesic dose of the selective μ agonist DAMGO, potentiates the intrathecal antinociception produced by either the selective agonist, DPDPE, or the selective κ_1 agonist, U5O,488H (Sutters et al., 1990). Intrathecal μ and κ opioid agonists administered simultaneously, as well as μ and δ opioid receptor agonists administered to spinal and supraspinal sites, each produce synergistic and supraadditive effects (Heyman et al., 1988).

3. It was also shown that coadministration of a low-analgesic dose of the selective μ agonist DAMGO, potentiates the intrathecal antinociception produced by either the selective δ agonist, DPDPE, or the selective κ agonist, U5O,488H (Sutters et al., 1990). Intrathecal μ and κ opioid agonists administered simultaneously, as well as μ and δ opioid receptor agonists administered to spinal and supraspinal sites, each produce synergistic and supraadditive effects (Heyman et al., 1988). Rossi and coworkers (1994) found that subthreshold doses of DAMGO coadministered simultaneously into the PAG and RVM produce a multiplicative analgesic interaction, implying μ/μ synergy. An interaction was also observed when DAMGO (μ agonist) was applied to one site and deltorphin II (δ_2 agonist) was applied to the second site, but not when DAMGO (μ agonist) was applied to one site and either U50488H (κ agonist) or DPDPE(δ_1 agonist) was applied to the second site. These data indicate the presence of μ_1/δ_2 interactions but the absence of μ/δ_1 or μ/κ_1 interactions.

Smith (1992) observed the occurrence of synergism when subthreshold doses of BEND and morphine are coadministered into the vlPAG. Using different μ opioid

antagonists, he suggested that BEND and morphine may function through separate and distinct receptor systems that may exist on separate inhibitory neuronal processes. Another possibility is that both of these agonists activate a single descending pathway by actions through a receptor complex with multiple sites for differential agonist binding (Monroe et al., 1996). No matter what the different mechanisms for BEND and morphine could be, a common theme in the synergy mechanism is that agonists ultimately have to functionally converge, either at supraspinal levels or at the level of dorsal horn neurons.

At the present time, the mechanism underlying antinociceptive synergy is unknown. Different lines of evidence suggest distinct explanations. For example, opioid agonists can act at different receptor sites, and that these two populations of opioid receptors, on the same or different neurons, interact to enhance each other's activity and produce a synergistic antinociceptive interaction. Consistent with this is the finding that demonstrated the presence of both μ- and δ-opioid receptors on primary nociceptive afferents and on second-order dorsal horn neurons in the rat (Fields et al., 1980). Also, δ, μ and κ receptors were observed on a single dorsal root ganglion (DRG) cell in vitro (Werz et al., 1987). Pick and Pasternak (1992) demonstrated that i.c.v. morphine potentiates a fixed low dose of i.t. morphine as effectively in μ-deficient CXBK mice as in the regular CD-1 mice, in spite of the fact that CXBK mice are insensitive to systemically-administered morphine. The μ_1-selective antagonist naloxonazine does not diminish the potency of i.c. v. morphine in the synergy model, but it blocks morphine antinociception following supraspinal administration alone. In contrast, B-FNA, which blocks both $\mu_1$and μ_2-opioid receptors, diminishes the potency of i.c.v morphine when

administered alone or when paired with i.t. morphine. Therefore, μ_2 receptors mediate both spinal analgesic responses and the synergism within spinal systems at the supraspinal level. Thus, the involvement and functional efficacies of receptors activated by certain combinations of ascending and descending pathways in the synergy model- that are dorment when only one route of administration, one drug or one site is activated- could very well explain the supra-additive effect of simultaneous application of drugs Some authors (Siuciak and Advokat, 1989; Fujimoto et al., 1988) suggested that the antinociceptive effect of i.t. morphine is tonically suppressed by descending inhibitory input. Supraspinal morphine removes this descending inhibition and allows the antinociceptive effect of spinal morphine to be expressed. This explanation is incompatible with the common view that supraspinal morphine increases descending inhibition of spinal nociceptive processing.

b. RVM antagonist effects upon morphine antinociception elicited from the vlPAG: Bodnar and colleagues (Kiefel et al., 1992a, 1992b, 1993) has employed an approach to identify the neurochemical substrates in the RVM mediating the antinociceptive effects of morphine microinjected into the vlPAG. Since 5-HT is found in 55-63% of the fibers between the vlPAG and RVM (Beitz, 1982a), Bodnar and colleagues (Kiefel et al., 1992a) confirmed that a serotonergic medullary synapse mediated morphine antinociception elicited from the vlPAG in that medullary methysergide, a general 5-HT receptor antagonist, reduced mesencephalic morphine antinociception on the tail-flick (69%) and jump (50%) tests. Two 5-HT receptor subtypes (5-HT_2, 5-HT_3) were implicated in this effect since mesencephalic morphine

antinociception was significantly reduced following RVM antagonist pretreatment with either ritanserin ($5\text{-}HT_2$) on the tail-flick (81%) and jump (65%) tests, and ICS205930 ($5\text{-}HT_3$) on the tail-flick (91%) and jump (63%) tests (Kiefel et al., 1992b). The specificity of such effects were verified by the failure of these antagonists to alter basal nociceptive thresholds following RVM administration. Further, medullary placements ventral or lateral to the RVM failed to support the antagonistic effects. Since enkephalin-immunoreactive neurons project from the vlPAG to the RVM (Beitz, 1982b) and also serve as intrinsic RVM interneurons (Lewis et al., 1985), Bodnar and colleagues (Keifel et al., 1993) found that mesencephalic morphine antinociception was significantly reduced following RVM antagonist pretreatment with naltrexone (general opioid), B-FNA (μ) and naltrindole (δ) opioid antagonists. Again these effects were specific based on the failure of these antagonists to alter basal nociceptive thresholds in the RVM and to alter mesencephalic morphine antinociception in sites ventral and lateral to the RVM.

E. Other Neurotransmitter Candidates for Antinociception in the RVM.

The antagonist approach will be used in the present studies to indentify further neurochemical substrates in the RVM mediating mesencephalic opioid antinociception. Two neurochemical receptor systems were chosen: 1.) EAA, and 2.) ACh.

1. Excitatory Amino Acid Receptors: Glutamate and aspartate are the principal excitatory neurotransmitters in the brain. EAAs and their receptors are recognized to exist in several distinct anatomical pathways, such as corticofugal projections, but their distribution is practically ubiquitous in the central nervous system (Greenamyre and

Porter, 1994). Consequently, they have been shown to participate in numerous normal and pathological neural processes, including nociception and antinociception.

EAA receptors are divided into ionotropic and metabotropic classes. The metabotropic subtypes (mGluR1-mGluR7) in turn have splice variants for the mGluR1, mGluR4 and mGluR5 receptors, further dividing those subtypes (Pin and Duvoisin, 1995). For example, the mGluR1 subtype has been further subdivied into mGluR1a, mGluR1b, mGluR1c, based on splice variants (Bockaert and Fagni, 1993). They consist of 7 transmembrane domains, a large extracellular region which binds ligands, and intracellular loops which interact with G-proteins (Pin and Duvoisin, 1995). These receptors are coupled via G-proteins to phosphoinositide hydrolysis, phospholipase D, and cAMP production. Production of cAMP may be increased (mGluR1a) or decreased (mGluR1b, mGluR2, mGluR3, and mGluR4), depending on the receptor subtype and intracellular mechanism. Further, they may indirectly modulate ion channels, including AMPA and NMDA receptor-channels (Schoepp and Conn, 1993). Based on amino acid sequence identity, they may be classified into 3 groups: group I is comprised of mGluR1 and mGluR5, group II is comprised of mGluR2 and mGluR3, and group III is comprised of mGluR4, mGluR6, and mGluR7 (Pin and Duvoisin, 1995). The activity in antinociception has yet to be investigated for mGluRs, although their role in nociception has been examined (Fisher and Coderre, 1996).

Ionotropic EAA receptors have been divided into three subtypes: N-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxazole-proprionic acid (AMPA), and kainate receptors (Nakanishi, 1992). The NMDA receptor-channel is a

cationic channel which, when open, allows influx of Ca^{2+} and Na^{+}, as well as efflux of K^{+}. In addition to a binding site for EAAs, the NMDA receptor-channel has several other ligand binding sites which modulate its function. Among them are the Mg^{2+} site which is located within the channel opening and blocks the channel in a voltage-dependent manner. The Zn^{2+} site is located extracellularly and also inhibits channel opening, but in a voltage-independent manner. Also within the channel opening is a binding site for MK-801, as well as the dissociative anesthetics phencyclidine and ketamine, where ligand binding inhibits ion flux as well. Thus, MK-801 acts as a non-competitive antagonist.

Binding sites which activate the channel include the NMDA receptor itself, which binds endogenous EAAs, and NMDA most selectively (Nakanishi, 1992). Competitive antagonists such as AP7 have been developed. Along with the NMDA site is a strychnine-insensitive glycine modulatory site, the agonism of which is essential for NMDA channel opening. Glycine is thus regarded as a co-agonist for the NMDA channel (Kleckner and Dingledine, 1988). Additional receptors on the NMDA receptor-channel are the polyamine binding sites, which bind endogenous polyamines such as spermine and spermidine and facilitate the activity of NMDA and glycine in opening the channel. However polyamines produce a voltage-dependent block of channel function at high concentrations, and some triamines, such as diethylenetriamine, act as antagonists at the polyamine receptors (Williams, Romano, Dichtern, and Molinoff, 1991; Rock and Macdonald, 1991). Additional factors which modify NMDA channel functioning are phosphorylation of select amino acid residues and pH, in which increasing alkalinity

facilitates channel opening (Traynelis and Cull-Candy, 1991).

The two remaining ionotropic EAA receptors are the AMPA and kainate receptors. The AMPA receptor is present on a cation channel which allows influx of Ca2+ and Na+. Pharmacologically, the AMPA receptor is distinct from the NMDA receptor in that NMDA recoginzes both glutamate and aspartate as agonists, but AMPA only recognizes glutamate. The kainate receptor is distinguishable from the AMPA receptor by rank-order of agonist potencies: kainate > glutamate > AMPA for kainate and AMPA > glutamate >kainate for AMPA.

Different functional roles of the ionotropic EAA receptors can be probed by using pharmacological antagonists. The functioning of the NMDA channel is antagonized by non-competetive channel blocking antagonist MK-801, as well as the competetive NMDA receptor antagonist (-)-2-amino-7-phosphonoheptanoate (AP7). Kainate and AMPA receptors are both antagonized by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX).

EAAs have been demonstrated to participate in supraspinal analgesic pathways since either L-glutamate or N-methyl-D-aspartate (NMDA) elicits antinociception following vlPAG (e.g., Behbehani and Fields 1979; Jacquet 1988; Siegfried and Nunes deSouza 1989) or RVM (Satoh et at. 1983b; Jensen and Yaksh 1984a,b; van Praag and Frenk 1990; McGowan and Hammond 1993a,b) administration. vlPAG neurons containing glutamate and aspartate (Clements et al. 1987) project to the RVM (Wiklund et at. 1988; Beitz 1990). Aimone and Gebhart (1986) found that EAA receptor antagonists administered into the RVM increased the intensity of electrical stimulation

elicited from the vlPAG. Further, vanPraag and Frenk (1990) found that morphine antinociception elicited from the vlPAG was significantly reduced by RVM pretreatment of the EAA antagonists, 1-(p-chlorobenzoyl)-piperazine-2,3dicarhoxylate (PCB) or DL-2-amino-5-phosphono-valerate (APV). Since PCB was more potent than APV in reducing vlPAG morphine antinociception, van Praag and Frenk (1990) suggested that kainate-quisqualate receptors modulated analgesic processes in the RVM more than NMDA receptors.

2. Acetylcholine Receptors. ACh has long been recognized as a neurotransmitter in the central nervous system. The major brainstem sources of cholinergic projections are the pedunculopontine and dorsolateral tegmental nuclei (Woolf and Butcher, 1989). Cholinergic receptors are grossly divided into nicotinic and muscarinic types, which are each further divided into subytypes. The nicotinic receptor is located on a ion channel, gating the flux of cations across the membrane. The channel is composed of 5 subunits, each with 4 transmembrane-spanning segments. The muscarinic receptors, in contrast, are G-protein linked receptors with 7 transmembrane domains. Five subtypes have been cloned, which create their intracellular effects by hydrolysis of phosphoinositides or inhibition of adenylate cyclase.

Muscarinic receptors have been implicated in cholinergic mechanisms of antinociception. The general cholinergic agonist, carbachol produces antinociception on the tail-flick test following microinjection into the NRM which is blocked by atropine, a muscarinic antagonist (Brodie and Proudfit, 1984, 1986), and also in both the parabrachial area (Hayes et al. 1984; Katayama et al., 1984a, 1984b; Menescal-de-

Oliveira and Hoffman, 1995) and the lateral reticular nucleus (Ossipov and Gebhart, 1986). Other sites supporting antinociception by carbachol are the dorsal and medial raphe, vlPAG, medial geniculate body, habenula, amygdala, hippocampus, hypothalamus and septum (Klamt and Prado, 1991). Carbachol-induced antinociception following injection into the dorsal and vlPAG is blocked by phenoxybenzamine (alpha-adrenergic) and mecamylamine (nicotinic), but not by naloxone, methysergide or atropine (Guimares and Prado, 1994).

Nicotinic receptors are also involved in cholinergic mechanisms of antinociception. Nicotine produces antinociception when administered into the vlPAG (Llewelyn et al., 1981). Nicotine in the PPTN or RVM increases hot-plate and tail-flick latencies, which is blocked by muscarinic receptors in both sites (Iwamoto, 1989, 1991). Recently, a high-potency nicotinic agonist has been developed, ABT-594, that has analgesic efficacy comparable to morphine across a number of nociceptive paradigms (Bannon, et al. 1998). Further, this agent did not appear to produce dependence or opioid-like withdrawal upon discontinuation.

Cholinergic analgesic mechanisms have shown to interact with opioid mechanisms. Cholinomimetics enhance morphine antinociception in animals (Bhargava and Way, 1972; Ireson, 1970; Lipman and Spencer, 1980), and produce antinociception following intracerebroventricular (Metys et al., 1969; Pedigo et al., 1975), intrathecal (Yaksh, 1985) and systemic (see review: Green and Kitchen, 1986; Harris et al., 1969; Tripathi et al., 1982) administration. Nicotine also produces antinociception following systemic treatment with opioids (Phan et al., 1973; Sahley and Berntson, 1979; Tripathi

et al., 1982). No interaction has been shown between crossed opioid and cholinergic antagonists (Green and Kitchen, 1986; Harris et al., 1969; Ireson, 1970). While scopolamine fails to block morphine antinociception (Ireson, 1970; Lipman and Spencer, 1980), atropine blocks this effect (Dirksen and Nijhuis, 1983; Lipman and Spencer, 1980). Systemic scopolamine potentiates systemic morphine and icv DADLE antinociception, but fails to affect i.c.v. BEND antinociception (Sperber et al., 1986).

The cholinergic system appears to exert a facilitatory effect upon opioid-mediated analgesic systems. Lesions placed in the pedunculopontine tegmental nucleus lesions failed to alter morphine antinociception on the formalin test (Olmstead and Franklin, 1993). Intrathecal administration of atropine reduced systemic morphine antinociception in intact, but not spinalized rats, suggesting a role for a descending cholinergic pathway (Chiang and Zhuo, 1989; Dirksen and Nijhuis, 1983). Spinal atropine also reduces stimulation-produced antinociception elicited from the NRGC (Zhou and Gebhart, 1990). In neurophysiological studies, cholinergic agonists in the RVM produce excitation (Behbehani, 1982; Duggan and Griersmith, 1979).

The RVM has cholinergic receptors (Kobayashi et al., 1978; Rotter et al., 1979) including muscarinic M_1 and M_2 subtypes (Cortes and Palacios, 1986; Nonaka and Moroji, 1984; Spencer et al., 1986; Wamsley et al., 1980) as well as nicotinic (London et al., 1985; Rainbow et al., 1984) receptors, with the latter in the dorsal raphe and surrounding vlPAG (Segal et al., 1978; Sofroniew et al., 1985). The RVM also contains choline acetyltransferase (Kobayashi et al., 1975) and acetylcholinesterase (Palkovits and Jacobowitz, 1974), indicating the presence of acetylcholine perikarya.

RATIONALE

Supraspinal opioid antinociception depends strongly in part by the activation of a descending output from the vlPAG to the RVM. These sites display the most marked supraspinal opioid synergy (Rossi et al., 1993), and the nature of the synergistic interactions involves μ/μ and μ/δ interactions between the vlPAG and RVM (Rossi et al., 1994). Kiefel and coworkers (1992a, 1992b, 1993) have validated the identification of neurochemical systems within the RVM mediating morphine antinociception elicited from the PAG by demonstrating that serotonergic (5-HT_2, 5-HT_3) and opioid (μ, δ) synapses in the RVM mediate mesencephalic morphine antinociception. The previous portions of the Background section provide convincing evidence for the hypotheses that EAA and cholinergic systems in the RVM should mediate morphine antinociception elicited from the vlPAG, and that antagonists of these receptor systems should reduce this response. This is the basis of the first two studies. Substantial evidence has been presented demonstrating marked dissociations between the analgesic responses elicited by morphine and BEND, particularly in the vlPAG. These data have led to the third hypothesis that BEND antinociception elicited from the vlPAG utilizes a pathway distinct from the RVM, and hence that EAA antagonists administered into the RVM should fail to alter this analgesic response. The three studies are as follows:

1. The first study explored the relationship between medullary EAA receptors in modulating morphine antinociception elicited from the vlPAG by evaluating whether RVM pretreatment of either the non-competitive NMDA antagonist, MK-801 ((+)-5-

methyl- 10,11 -dihydro-5H-dibenzo (a,d) cyclohepten-5,10-imine maleate), the competitive NMDA antagonist, AP-7 ((-)-2-amino-7-phosphonoheptanoate) or the kainate/AMPA antagonist CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), alter antinociception on the tail-flick and jump tests following morphine administered into the vlPAG. Since NMDA antagonists may potentially exert their effects by diffusing into structures outside of the injection area (Nasstrom et al. 1993a,b), additional groups of rats with misplaced medullary cannulae aimed dorsal and lateral to the RVM will be tested in the same paradigm. This experiment was published in the journal Pain in 1996 (Spinella et al., 1996).

2. The second study assessed the role of muscarinic and nicotinic cholinergic receptors in the RVM in the mediation of morphine antinociception elicited from the vlPAG by evaluating whether RVM pretreatment of either muscarinic (scopolamine), M_1 (pirenzepine), M_2 (methoctramine), or nicotinic (mecamylamine) cholinergic receptor antagonists altered antinociception on the tail-flick and jump tests in rats following morphine administration into the vlPAG. The present study will also evaluate whether cholinergic antagonists in the RVM alter basal nociceptive latencies and thresholds to assess whether the antinociceptive changes were due to corresponding hyperalgesic effects of the antagonists. Finally, to ascertain whether the anatomical specificity of cholinergic antagonist effects was limited to the RVM, additional groups of rats with misplaced medullary cannulae aimed dorsal and lateral to the RVM will be tested in the same paradigm. This experiment was published in the journal, Analgesia in 1997 (Spinella et al., 1997).

3. The third study will assess the interaction of BEND with EAA and cholinergic receptors in the RVM by evaluating whether RVM pretreatment of either the noncompetitive (MK-801) or competitive (AP7) NMDA antagonists or muscarinic (scopolamine) or nicotinic (mecamylamine) antagonists alter antinociception on the tail-flick and jump tests in rats following BEND administration into the vlPAG. Finally, to confirm that antinociception produced by BEND in the vlPAG operates through an opioid synapse, additional animals will be tested for BEND antinociception BEND administration in the vlPAG following vlPAG pretreatment with the general opioid antagonist, naltrexone. This experiment has been submitted for publication in 1998.

II. GENERAL METHODS

Subjects. Adult male albino Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA; 80-120 days of age) were housed individually and maintained on a 12-h light/dark cycle with food and water available ad libitum.

Surgery and Histology. Following anesthesia with chlorpromazine (3 mg/kg, ip.) and ketamine HCI (100 mg/kg, i.m.), two stainless steel guide cannulae (26 gauge, Plastics One, Roanoke, VA) were placed stereotaxically (Kopf Instruments) into the vlPAG and RVM of rats (Bodnar et al. 1988; Kiefel et al. 1992a,b, 1993). Stereotaxic coordinates were: incisor bar (-5 mm), vlPAG: 0.3-0.6 mm anterior to the lambda suture, 1.5-2.0 mm lateral to the sagittal suture, 6.5-7.0 mm from the top of the skull, and angled towards the sagittal suture at 12°, and RVM: 10.8-11.3 posterior to the bregma suture, 0-0.7 mm lateral to the midline, and 10.0-11.0 mm from the top of the skull. Additional control groups in experiments 1 and 2 were stereotaxically implanted with cannuale aimed at the vlPAG 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. Cannulae were secured to anchor screws with dental acrylic. All animals were allowed 1 week to recover and clear anesthetic. After testing, cannulae placements were histologically examined in anesthetized (Euthanasia, H. Schein) rats that received a trans-cardiac perfusion with 0.9% normal saline followed by 10% buffered formalin. Coronal (40 um) sections, stained with cresyl violet were examined by light microscopy by an observer unfamiliar with the behavioral data. Only animals with confirmed cannulae placements were included in the data analyses.

Nociceptive tests. In a given session, each animal was tested on the tail-flick and jump test in that order to minimize carryover effects between tests. A tail-flick analgesiometer (IITC) produced a radiant heat source that was mounted 8 cm above a photocell upon which the rat's tail was placed. Radiant heat was applied 3-9 cm proximal to the tip of the rat's tail; removal of the tail activated the photocell and determined the latency (0.01 sec accuracy). The thermal intensity of the radiant heat source was set to produce baseline tail-flick latencies between 2 and 3.5 sec. Each session consisted of 3 latency determinations at different points on the tail at 10-sec intertrial intervals. To avoid tissue damage, a trial was automatically terminated if a response did not occur within 12 sec. Rats were tested on the jump test immediately after tail-flick testing. Electric shock was delivered to the feet of the rat by a shock generator (BRS/LVE) and shock scrambler (Campden Instruments). The jump threshold was defined in milliamperes (mA) as the lowest of two consecutive ascending intensities in which the animal simultaneously removed both hindpaws from the grids. Each of six trials began with the animal receiving a 300-msec footshock at a current intensity of 0.10 mA with subsequent shocks increased in 0.05 mA steps at 10-sec intervals until the jump threshold was determined. Baseline latencies and thresholds were determined for at least 4 days before experimental testing began to insure stability of responding. All animals displayed consistent latencies and thresholds in baseline and vehicle testing that did not appear subject to desensitization.

Drugs and Injections. All microinfusions were administered in 1ul volumes at a rate of 0.2 ul every 10 sec through a stainless steel internal cannula (33 gauge, Plastics

One) which was connected to a Hamilton microsyringe by polyethylene tubing. The internal cannula was left in place for at least 30 s after injection to minimize suction of the injectate through the guide cannula following removal of the internal cannula. Medullary microinjections of the antagonists preceded mesencephalic microinjections by 20 min. Morphine (Pennick Laboratories), MK-801 (Research Biochemicals, MW = 383.7) and AP7 (Research Biochemicals, MW = 225.2) were dissolved in normal saline. CNQX (Research Biochemicals, MW = 232.2) was dissolved in 45% (w/v) aqueous 2-hydroxypropyl-cyclodextrin buffer (Research Biochemicals) in distilled water. B-END (Peninsula Laboratories, MW=3465), scopolamine hydrobromide (Research Biochemicals, MW = 384.3), pirenzepine dihydrochloride (Research Biochemicals, MW = 424.3), metboctramine tetrahydrochloride (Research Biochemicals, MW = 728.8), and mecamylamine hydrochloride (Research Biochemicals, MW = 203.8) were dissolved in distilled water.

Statistical analyses. Analyses of variance indicated that significant differences in latencies and thresholds failed to occur between baseline and vehicle-vehicle conditions in all experiments (data not shown); the latter were used for subsequent comparisons with agonist and antagonist effects. Separate split-plot analyses of variance were performed for each of the antagonist and control protocols. Each split-plot analysis of variance assessed significant effects among conditions (between-groups factor) and across the time course (within-groups factor) with Dunnett comparisons ($p<0.05$) determining opioid agonist antinociception relative to vehicle/vehicle treatment. Dunn comparisons ($p<0.05$) determined antagonist effects relative to vehicle/opioid agonist

treatment.

III. EXPERIMENT 1

A. Introduction: EAAs have been demonstrated to participate in supraspinal analgesic pathways since either L-glutamate or N-methyl-D-aspartate (NMDA) elicits antinociception following vlPAG (e.g., Behbehani and Fields 1979; Jacquet 1988; Siegfried and Nunes deSouza 1989) or RVM (Satoh et at. 1983b; Jensen and Yaksh 1984a,b; van Praag and Frenk 1990; McGowan and Hammond 1993a,b) administration. vlPAG neurons containing glutamate and aspartate (Clements et al. 1987) project to the RVM (Wiklund et at. 1988; Beitz 1990). Aimone and Gebhart (1986) found that EAA receptor antagonists administered into the RVM increased the intensity of electrical stimulation antinociception elicited from the vlPAG. Further, vanPraag and Frenk (1990) found that morphine antinociception elicited from the vlPAG was significantly reduced by RVM pretreatment of the EAA antagonists, 1-(p-chlorobenzoyl)-piperazine-2,3dicarhoxylate (PCB) or DL-2-amino-5-phosphono-valerate (APV). Since PCB was more potent than APV in reducing vlPAG morphine antinociception, van Praag and Frenk (1990) suggested that kainate-quisqualate receptors modulated analgesic processes in the RVM more than NMDA receptors. It should be noted however that Bodnar and colleagues was working with more selective and potent antagonists for the NMDA (AP7 and MK-801) and AMPA/kainate (CNQX) receptor subtypes.

B. Protocols. Separate groups of rats received a maximum of five pairs of microinjection conditions at weekly intervals. Tail-flick latencies and jump thresholds were determined at 30, 60, 90 and 120 mm following the second microinjection of each pair. Table 1 summarizes the treatments, doses and sample sizes used to assess the effects of either MK-801 (A), AP7 (B) or CNQX (C) administered into the RVM upon morphine

antinociception elicited from the vlPAG, while the D section summarizes treatments of control rats with medullary placements lateral and dorsal to the RVM. Higher doses of CNQX could not be used because of solubility problems and limitations to a 1 μl volume. Animals receiving particular antagonist doses were matched on the basis of their basal nociceptive latencies and thresholds as well as the degree of mesencephalic morphine antinociception. A morphine dose of 2.5 ug was chosen because of its significant, but not maximal analgesic effects following vlPAG microinjection, and the weekly interval between conditions minimized possible tolerance effects (e.g. Bodnar at al., 1988; Kiefel et al, 1992a,b, 1993).

C. Results.

1. Histological Verifications. In the present and subsequent experiments, mesencephalic cannula placements were all localized in the lateral, ventral and ventrolateral quadrants of the vlPAG and immediately adjacent tegmentum as far rostral as the III cranial nerve nucleus and as far caudal as the dorsal raphe nucleus. Figure 1a displays such vlPAG placements. 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. Misplaced medullary cannula placements were localized along the rostro-caudal extent of RVM placements, but were located lateral and dorsal to this area. Figure 1b displays the proper and misplaced RVM placements respectively. There were both anatomical overlap and similar analgesic potency for mesencephalic cannula placements of rats with RVM and misplaced medullary cannula placements.

Figure 1 Schematic representation of cannula placement in a) vlPAG and b) RVM, as confirmed by histological verification using the atlas of Paxinos and Watson (1968). a) Mesencephalic placements were in the lateral, ventral, and ventrolateral PAG, as far caudal as the dorsal raphe nucleus, b) appropriate RVM placements were localized in either the NRM, NRGC or NRGC pars alpha. Control misplacements were located lateral and dorsal to this area.

Figure 1a

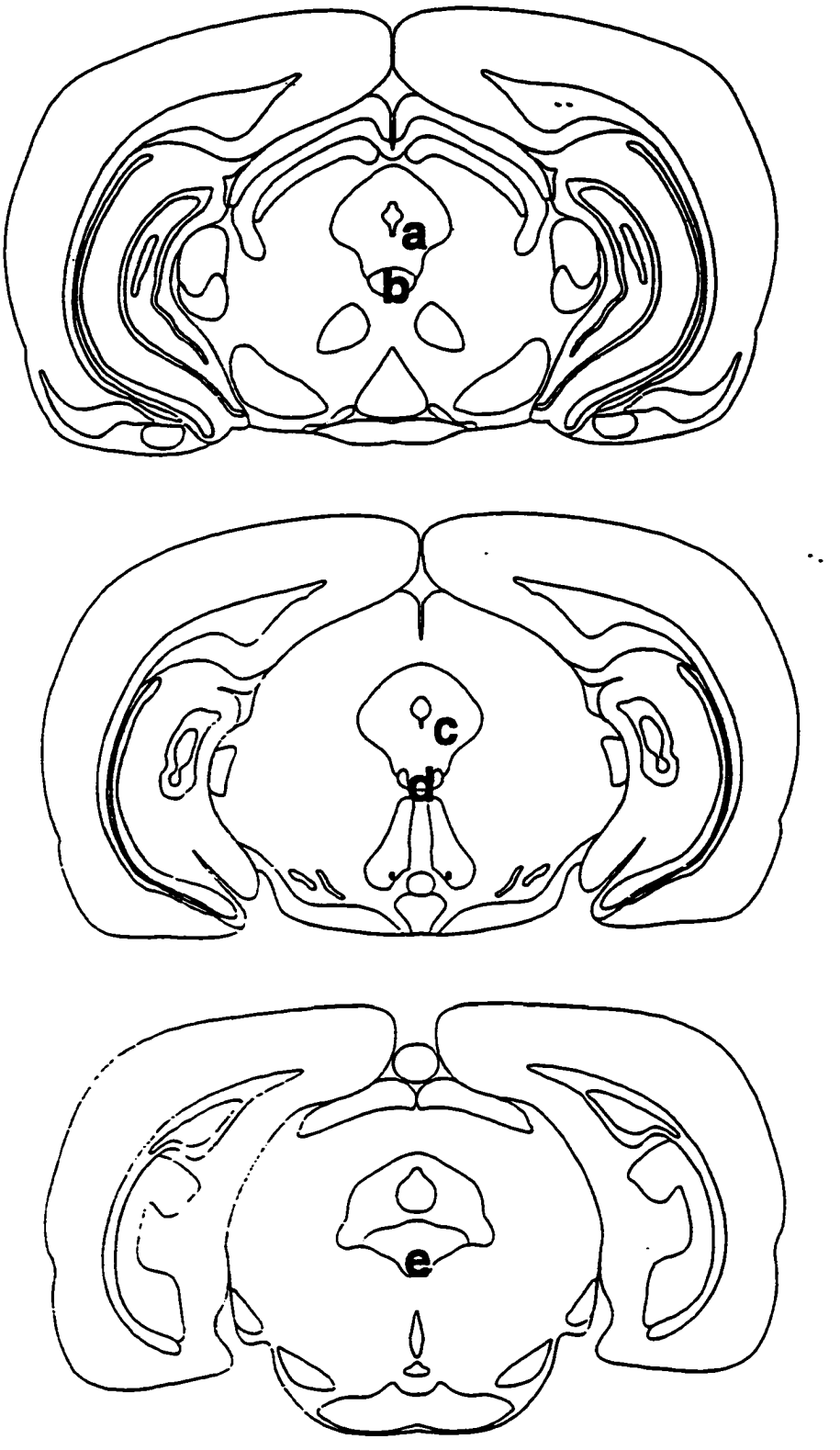
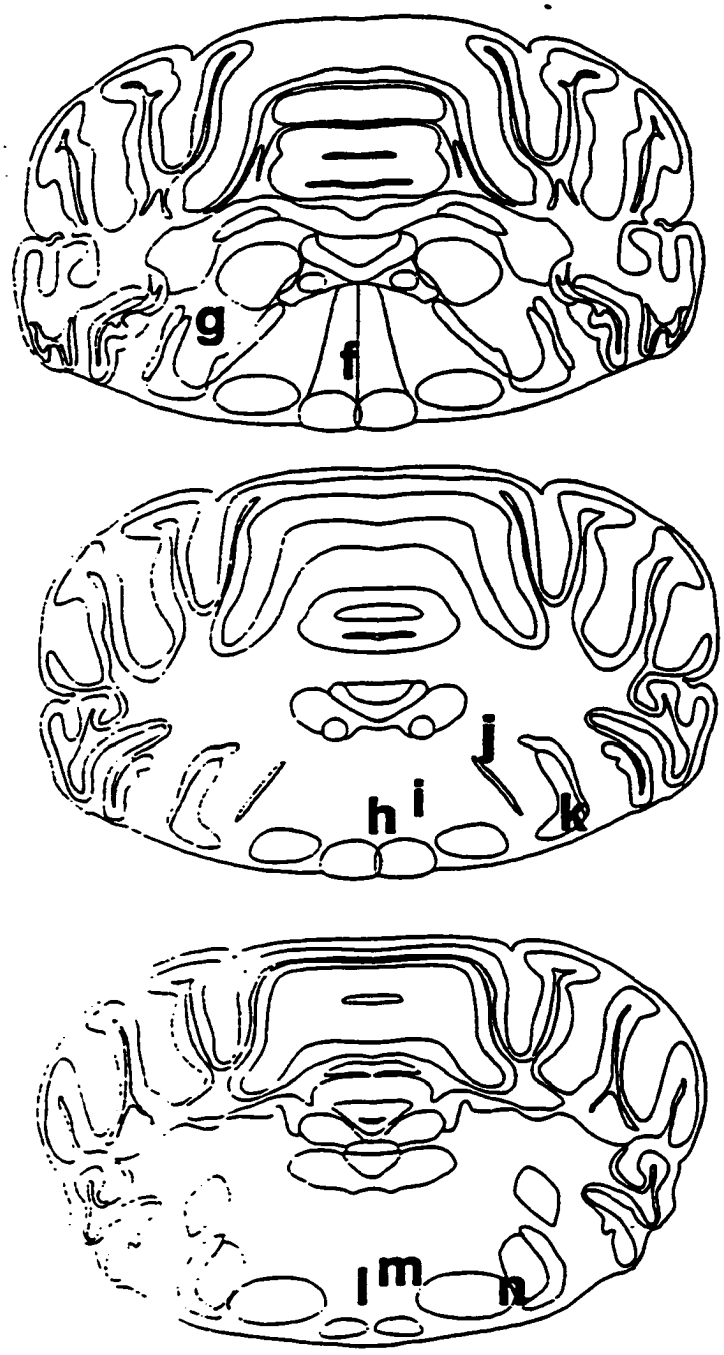


Figure 1b



2. RVM MK-801 and mesencephalic morphine antinociception: Significant differences were observed among conditions (tail-flick: $F_{5,30} = 20.65$, $p < 0.0001$; jump: $F = 12.02$, $p < 0.0001$), across test times (tail-flick: $F3,90 = 19.57$, $p < 0.0001$; jump: $F = 1.60$, ns) and for their interaction (tail-flick: $F_{15,90} = 4.09$, $P < 0.0001$; jump: $F = 3.94$, $p < 0.0001$). Morphine in the vlPAG significantly increased latencies (30-120 min, Fig. 2, upper panel) and thresholds (30-90 min, Fig. 2, lower panel). In rats receiving RVM microinjections of the lowest (0.03 μg, 0.08 nmol) and highest (3 μg, 7.8 nmol) MK-801 doses, mesencephalic morphine antinociception was significantly reduced across the time course on the tail-flick test to within vehicle-vehicle levels. RVM microinjections of the middle (0.3 μg, 0.8 nmol) MK-801 dose significantly reduced mesencephalic morphine antinociception only after 30 min on the tail-flick test. All MK-801 doses in the RVM significantly reduced mesencephalic morphine antinociception across the time course on the jump test to within the range of vehicle-vehicle values. MK-801 (3 μg) in the RVM significantly decreased basal jump thresholds (30-60 mm), but not basal jump test latencies relative to vehicle-vehicle values.

3. RVM AP7 and mesencephalic morphine antinociception: Significant differences were observed among conditions (tail-flick: $F_{5,48} = 9.64$, $p < 0.0001$; jump: $F = 30.14$, $p < 0.0001$), across test times (tail-flick: $F_{3,144} = 42.11$, $p < 0.0001$; jump $F = 26.51$, $p < 0.0001$) and for their interaction (tail-flick: $F_{15,144} = 7.57$, $p < 0.0001$; jump: $F = 10.13$, $p < .0001$). Morphine in the vlPAG significantly increased latencies (Fig. 3A) and thresholds (Fig. 3B) for up to 90 min. In rats receiving RVM microinjections of the middle (0.1 μg, 0.4 nmol) and highest (1 μg, 4.4 nmol) AP7 doses, mesencephalic

TABLE 1

EXPERIMENT 1: SUMMARY OF EXPERIMENTAL GROUPS

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

RVM condition	PAG condition	n
A. MK-801 group		
Vehicle	Vehicle	6
Vehicle	Morphine (2.5 ug)	6
MK-801 (0.03 ug)	Morphine (2.5 ug)	6
MK-801 (0.3 ug)	Morphine (2.5 ug)	6
MK-801 (3.0 ug)	Morphine (2.5 ug)	6
MK-801 (3.0 ug)	Vehicle	6
B. AP7 group		
Vehicle	Vehicle	9
Vehicle	Morphine (2.5 ug)	9
AP7 (0.01 ug)	Morphine (2.5 ug)	8
AP7 (0.1 ug)	Morphine (2.5 ug)	8
AP7 (1.0 ug)	Morphine (2.5 ug)	9
AP7 (1.0 ug)	Vehicle	8
C. CNOX group		
Vehicle	Vehicle	6
Vehicle	Morphine (2.5 ug)	6
CNQX (0.5 ug)	Morphine (2.5 ug)	6
CNQX (0.5 ug)	Vehicle	6
D. Control group		
Vehicle	Vehicle	5
Vehicle	Morphine (2.5 ug)	5
MR-SO) (3 ug)	Morphine (2.5 ug)	5
AP7 (1 ug)	Morphine (2.5 ug)	5

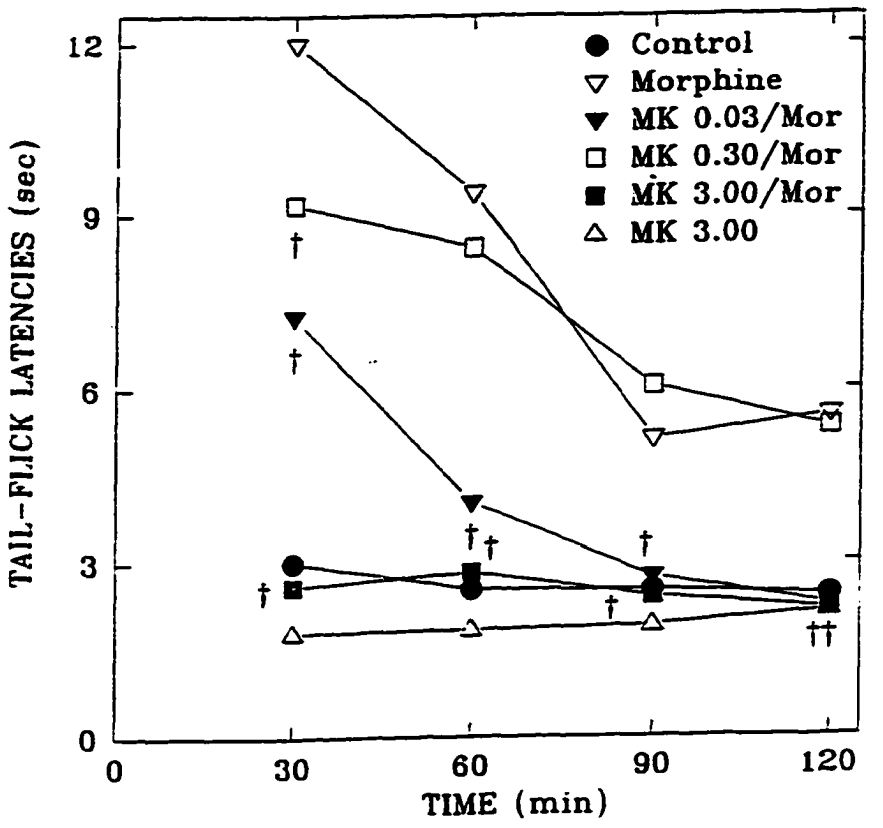
morphine antinociception was significantly reduced for up to 60 min on the tail-flick test; the low (0.01 μg, 0.04 nmol) AP7 dose failed to exert effects. Whereas RVM microinjections of the middle and highest AP7 doses significantly reduced mesencephalic morphine antinociception across the time course on the jump test, the low AP7 dose significantly reduced mesencephalic morphine antinociception only after 90 and 120 min. RVM microinjections of AP7 failed to alter basal latencies or thresholds.

4. RVM CNQX and mesencephalic morphine antinociception: Significant differences were observed among conditions (tail-flick: $F_{3,20} = 41.94$, $p < 0.0001$; jump: $F = 42.13$, $p < 0.0001$), across times (tail-flick: $F_{3,60} = 32.78$, $p < 0.0001$; jump: $F = 15.46$, $p < 0.0001$) and for their interaction (tail-flick: $F_{9,60} = 10.80$, $p < 0.0001$; jump: $F = 8.58$, $p < 0.0001$). Morphine in the vlPAG significantly increased latencies (30-90 min, Figure 4, upper panel) and thresholds (30-120 min, Figure 4, lower panel). While RVM microinjections of CNQX (0.5 μg, 2.2 nmol) failed to alter mesencephalic morphine antinociception on the tail-flick test, it produced small but significant reductions in mesencephalic morphine antinociception for up to 90 min on the jump test.

5. Mesencephalic morphine antinociception and NMDA antagonists in control placements: Significant differences were observed among conditions (tail-flick: $F_{3,16} = 37.29$, $p < 0.0001$; jump: $F=17.40$, $p < 0.0001$), across times (tail-flick: $F_{3,48} = 88.93$, $p < 0.0001$; jump: $F = {}_{45,98}$, $p < 0.0001$) and for their interaction (tail-flick: $F_{9,48} = 10.74$, $p < 0.0001$; jump: $F = {}_{5,25}$, $p < 0.0001$). Morphine in the vlPAG significantly increased latencies (30-90 min) and thresholds (30-120 min) (Table 2). Microinjections of the highest MK-801 or AP7 doses into misplaced medullary sites failed to significantly alter

Figure 2 EXPERIMENT 1: RVM MK801 and Mesencephalic Morphine Antinociception. Alterations in tail flick latencies (top panel) and jump test thresholds (lower panel) in rats injected with 2.5 ug morphine in the vlPAG and MK-801 (0.03, 0.3, 3.0 ug) in the RVM..

Figure 2



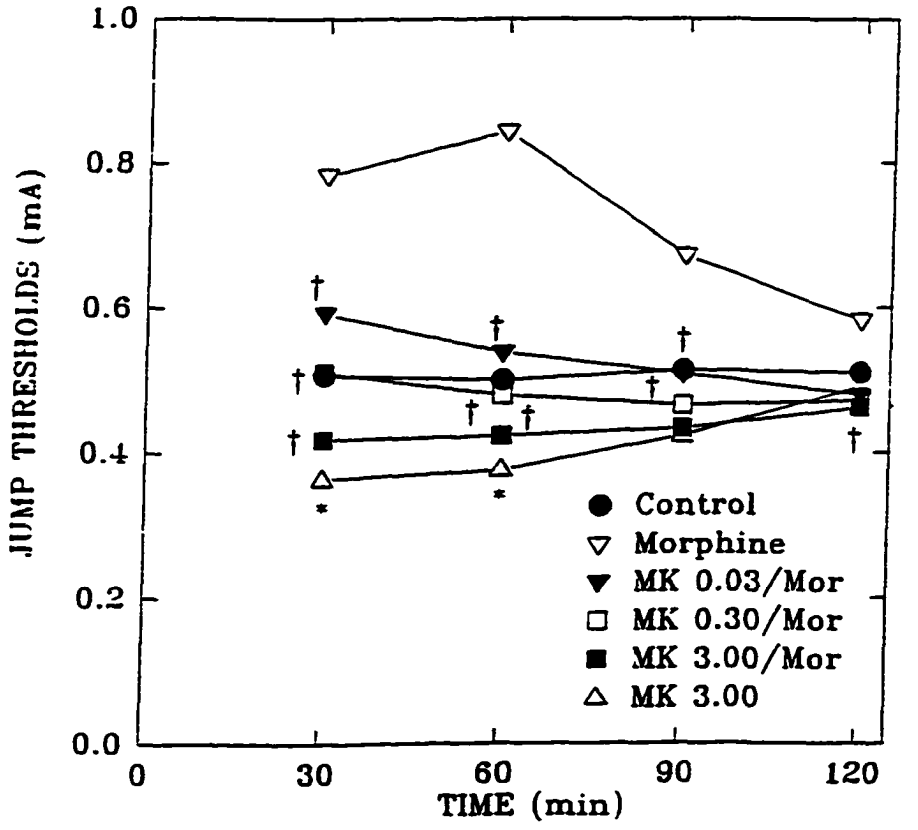
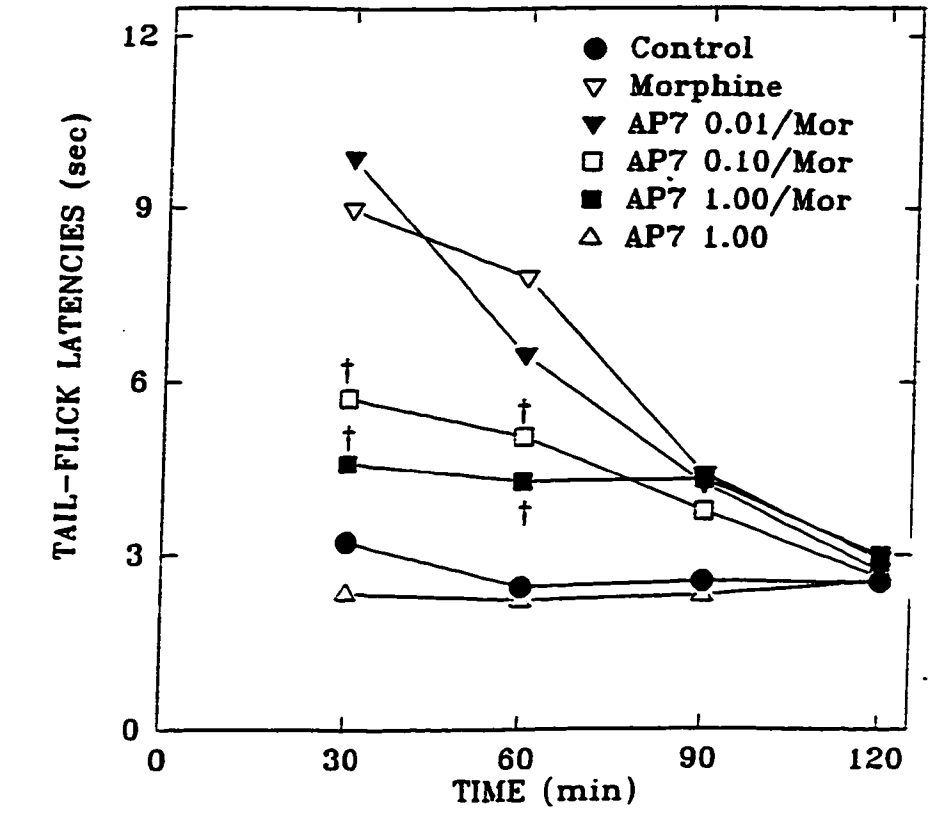


Figure 3 EXPERIMENT 1: RVM AP7 and Mesencephalic Morphine Antinociception. Alterations in tail flick latencies (top panel) and jump test thresholds (lower panel) in rats injected with 2.5 ug morphine in the vlPAG and AP7 (0.01, 0.1, 1.0 ug) in the RVM.

Figure 3



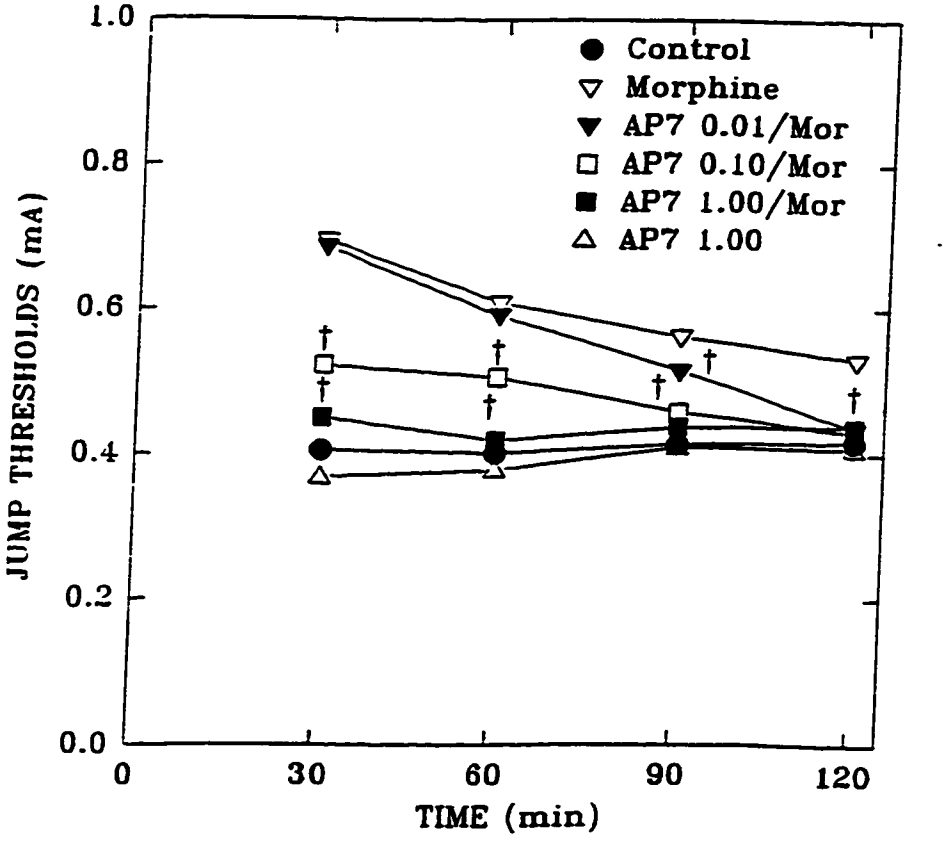
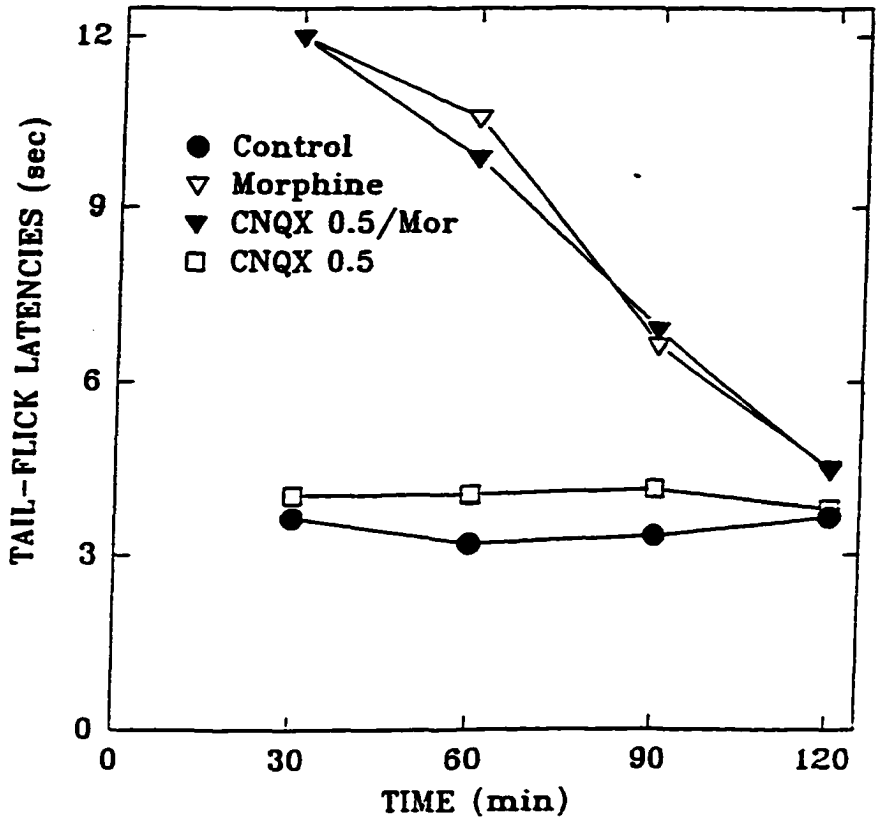


Figure 4 EXPERIMENT 1: RVM CNQX and Mesencephalic Morphine Antinociception. Alterations in tail flick latencies (top panel) and jump test thresholds (lower panel) in rats injected with 2.5 ug morphine in the vlPAG and CNQX (0.5 ug) in the RVM.

Figure 4



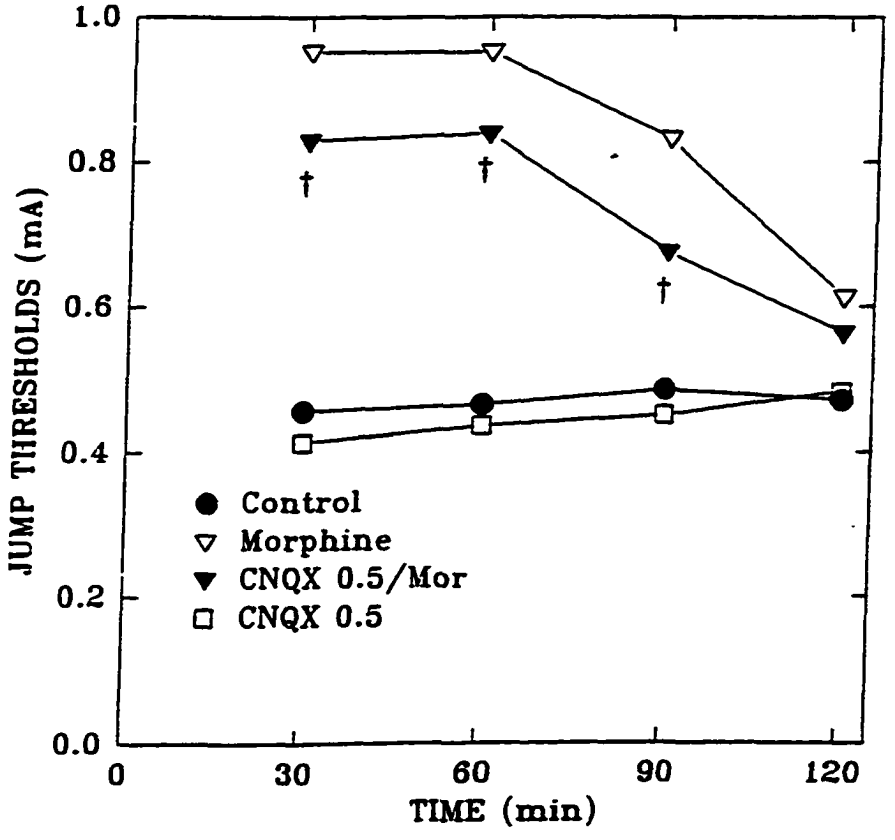


TABLE 2

EXPERIMENT 1: Alterations in Mesencephalic Morphine Analgesia by Preeatment of Either MK-801 or AP7 in Medullary Sites Lateral and Dorsal to the Rostral Ventromedial Medulla.

Veh -Vehicle; Mor-Morphine (2.5 ug); MK-801(3 ug); AP7 (1 ug). The first injection was administered into the misplaced medullary placement and the second injection was administered into the periaqueductal gray.

Condition	Post-injection time (mm)			
	30	60	90	120
Tail-flick latencies (sec)				
VehyVeh	3.25	2.64	3.12	3.05
Veh/Mor	12.00*	10.51*	7.29*	3.75
MK-801/Mor	12.00*	11.24*	6.47*	4.88*
AP7/Mor	12.00*	10.80*	7.82*	4.41
Jump Thresholds (mA)				
Veh/Veh	0.363	0.338	0.343	0.358
Veh/Mor	0.930*	0.828*	0.703*	0.608*
MK-801/Mor	0.785* +	0.726* +	0.583* +	0.525* +
AP7/Mor	0.833* +	0.750*	0.600* +	0.507* +

* Significant difference relative to corresponding Veh/Veb (Dunnett comparison, $P < 0.05$).

\+ Significant difference relative to corresponding Veh/Mor (Dunn comparison, $P < 0.05$).

mesencephalic morphine antinociception on the tail-flick test, but produced small, but significant reductions in antinociception on the jump test.

D. Discussion.

The present study found that RVM administration of either non-competitive (MK-801) or competitive (AP7) NMDA antagonists significantly inhibited mesencephalic morphine antinociception. Mesencephalic morphine antinociception was eliminated by MK-801 on both tests, and was potently reduced by AP7. These effects were highly selective for three reasons. First, if either MK-801 or AP7 was microinjected into misplaced medullary cannula placements that were lateral and dorsal to the RVM, they failed to alter mesencephalic morphine antinociception on the tail-flick test, and produced far smaller, but significant reductions on the jump test. This control suggests that the inhibitory actions of MK-801 and AP7 upon mesencephalic morphine antinociception are acting largely at the RVM injection sites, and not non-specifically through diffusion (e.g., see Nasstrom et al. 1993a,b). Second, RVM microinjections of the highest MK-801 dose failed to alter basal latencies, and only produced small, but significant reductions in basal thresholds. However, the magnitude of this hyperalgesia could not account for the observed marked reductions in mesencephalic morphine antinociception. Further, RVM microinjections of the highest AP7 dose failed to alter basal latencies or thresholds. These data indicate that the reductions in mesencephalic morphine antinociception were not the result of offsetting hyperalgesic properties of NMDA antagonists in the RVM. Third, RVM microinjection of the AMPA/kainate antagonist, CNQX failed to alter mesencephalic morphine antinociception on the tail-

flick test, and produced only small, significant reductions in mesencephalic morphine antinociception on the jump test. However, the CNQX effects were far smaller than competitive or non-competitive NMDA antagonist effects even though the doses were of comparable molecular weights. This control suggests specificity in of NMDA receptor antagonist effects in the RVM upon mesencephalic morphine antinociception.

Administration of either L-glutamate or aspartate elicits antinociception from several brain sites, including the vlPAG (Behbehani and Fields 1979; Jacquet 1988; Siegfried and Nunes de Souza 1989) and RVM (Satoh et al. 1983b; Jensen and Yaksh 1984a,b; vanPraag and Frenk 1990; McGowan and Hammond 1993a,b). Further, a subset of glutamate-containing and aspartate-containing cells in the vlPAG (Clements et al. 1987) project to the RVM (Wiklund et al. 1988; Beitz 1990). EAA transmitters and receptors modulate analgesic responses both within and between the vlPAG and RVM. Whereas antinociception elicited by vlPAG NMDA was reduced by vlPAG AP7 (Jacquet 1988), antinociception elicited by vlPAG glutamate was reduced by RVM PCB, but not RVM APV (vanPraag and Frenk 1990). Intracerebroventricular (i.c.v.) administration of MK-801 and CNQX also produce differential effects upon different forms of i.c.v. opioid antinociception in mice (Suh et al. 1995a). Whereas i.c.v. MK-801 blocked antinociception elicited by i.c.v. μ (morphine), κ_1 (U50488H) and δ_1 (D-Pen^2, D-Pen^5-enkephalin: DPDPE) opioid agonists, it did not block B-END antinociception. In contrast, i.c.v. CNQX only blocked morphine antinociception, but not antinociception elicited by either U50488H, DPDPE or BEND.

VanPraag and Frenk (1990) previously demonstrated that RVM administration of

either PCB or APV significantly reduced vlPAG morphine antinociception. Since PCB was more potent on a molar basis than APV in exerting these effects, and since PCB, but not APV blocked RVM glutamate antinociception, it appeared that the kainate/ quisqualate receptor subtype was primarily responsible for EAA modulation of analgesic responses in the RVM. The present study came to a different conclusion based upon the potent effectiveness of either competitive (AP7) or non-competitive (MK-801) NMDA receptor antagonists in the RVM to block vlPAG morphine antinociception as compared to the relative ineffectiveness of RVM microinjections of CNQX, a selective AMPA/ kainate antagonist. There were several procedural and methodological differences between studies. vanPraag and Frenk (1990) utilized a high (10 μg) dose of morphine in the vlPAG that elicited maximal antinociception at a short (7 sec) cut-off latency immediately after opiate administration. In contrast, Bodnar and colleagues used a smaller (2.5 μg) dose of morphine antinociception in the vlPAG that produced pronounced, yet submaximal antinociception (Bodnar et al. 1988; Smith et al. 1988; Kiefel et at. 1992a,b, 1993; Rossi et at. 1993, 1994; Urban and Smith 1993, 1994). Further, we used two nociceptive tests with higher cut-off latencies and thresholds across a far longer (120 min) time course. Whereas the former procedure yields maximal antinociception that may be somewhat impervious to physiological manipulations, the latter procedure yields potent antinociception which can be more sensitive to alterations induced by physiological manipulations (e.g., Kiefel et at. 1992a,b, 1993; Urban and Smith 1993). This is consistent with the dose range of EAA antagonists employed in the two studies. Whereas μmol doses of PCB and APV in the RVM were needed to elicit

reductions of 50-70% of vlPAG morphine (10 μg) antinociception (vanPraag and Frenk 1990), low nmol doses of MK-801 and AP7 in the RVM were capable of virtually eliminating vlPAG morphine (2.5 μg) antinociception. Further, these far lower doses of EAA antagonists failed to exert effects when the medullary placement was dorsal and lateral to the RVM. It is unknown whether diffusion factors of the far higher EAA antagonist doses (see Nasstrom et at. 1993a,b) may have produced some of the effects found by vanPraag and Frenk (1990). Given these provisos, it appears that EAA transmitters and receptors modulate supraspinal analgesic processes in general, and that NMDA receptors in the RVM modulate morphine antinociception elicited from the vlPAG in particular.

The integrity of the RVM is important for the full expression of analgesic responses elicited from the vlPAG based upon lesion studies (Behbehani and Fields 1979; Mohrland et al. 1982; Prieto et al. 1983; Morton et al. 1984), and administration of reversible neuronal blocking agents (Proudfit 1980; Gebhart et al. 1983; Sandkuhler and Gebhart 1984; Urban and Smith 1994). The neurochemical substrates mediating mesencephalic morphine antinociception in the RVM are complex, and include GABA (Heinricher et al. 1991; McGowan and Hammond 1993a,h), serotonin (Kiefel et al. 1992a,b), opioids (Heinricher et al. 1992, 1994; Morgan et al. 1992; Kiefel et al. 1993) and neurotensin (Urban and Smith 1993). The present and previous (Aimone and Gebhart 1986; vanPraag and Frenk 1990) studies indicate that EAA transmitters and receptors in the RVM are involved in the modulation of opioid antinociceptive processing from the vlPAG as well.

IV. EXPERIMENT 2

A. Introduction. Muscarinic receptors have been implicated in cholinergic mechanisms of antinociception. The general cholinergic agonist, carbachol produces antinociception on the tail-flick test following microinjection into the NRM which is blocked by atropine, a muscarinic antagonist (Brodie and Proudfit, 1984, 1986). Carbachol-produced antinociception following injection into the dorsal and ventral vlPAG is blocked by phenoxybenzamine (alpha-adrenergic) and mecamylamine (nicotinic), but not by naloxone, methysergide or atropine (Guimares and Prado, 1994). Nicotinic receptors are also involved in cholinergic mechanisms of antinociception. Nicotine produces antinociception when administered into the vlPAG (Llewelyn et al., 1981). Nicotine in the PPTN or RVM increases hot-plate and jump test latencies, which is blocked by muscarinic receptors in both sites (Iwamoto, 1989, 1991).

Cholinergic analgesic mechanisms have shown to interact with opioid mechanisms. Cholinomimetics enhance morphine antinociception in animals (Bhargava and Way, 1972; Ireson, 1970; Lipman and Spencer, 1980), and produce antinociception following intracerebroventricular (Metys et al., 1969; Pedigo et al., 1975), intrathecal (Yaksh, 1985) and systemic (see review: Green and Kitchen, 1986; Harris et al., 1969; Tripathi et al., 1982) administration. Nicotine also produces antinociception following systemic treatment with opioids (Phan et al., 1973; Sahley and Berntson, 1979; Tripathi et al., 1982). No interaction has been shown between crossed opioid and cholinergic antagonists (Green and Kitchen, 1986; Harris et al., 1969; Ireson, 1970). While scopolamine fails to block morphine antinociception (Ireson, 1970; Lipman and Spencer,

1980), atropine blocks this effect (Dirksen and Nijhuis, 1983; Lipman and Spencer, 1980). Systemic scopolamine potentiates systemic morphine and icv DADL antinociception, but fails to affect i.c.v. BEND antinociception (Sperber et al., 1986).

The cholinergic system appears to exert a facilitatory effect upon opioid-mediated analgesic systems. Lesions of the pedunculopontine tegmental nucleus lesions fail to alter morphine antinociception on formalin test (Olmstead and Franklin, 1993). Intrathecal administration of atropine reduces systemic morphine antinociception in intact, but not spinalized rats suggesting a role for a descending cholinergic pathway (Chiang and Zhuo, 1989; Dirksen and Nijhuis, 1983). Spinal atropine also reduces stimulation-produced antinociception from the NRGC (Zhou and Gebhart, 1990). In neurophysiological studies, cholinergic agonists in RVM produce excitation (Behbehani, 1982; Duggan and Griersmith, 1979).

The RVM has cholinergic receptors (Kobayashi et al., 1978; Rotter et al., 1979) including muscarinic M_1 and M_2 subtypes (Cortes and Palacios, 1986; Nonaka and Moroji, 1984; Spencer et al., 1986; Wamsley et al., 1980) as well as nicotinic (London et al., 1985; Rainbow et al., 1984) receptors, with the latter in the dorsal raphe and surrounding vlPAG (Segal et al., 1978; Sofroniew et al., 1985). The RVM also contains choline acetyltransferase (Kobayashi et al., 1975) and acetylcholinesterase (Palkovits and Jacobowitz, 1974), indicating the presence of acetylcholine perikarya.

B. Protocols. Separate groups of rats received a maximum of five pairs of microinjection drug conditions at weekly intervals. Latencies and thresholds were determined at 30, 60, 90, and 120 min following the second microinjection of each pair.

TABLE 3

EXPERIMENT 2: SUMMARY OF EXPERIMENTAL GROUPS

RVM Condition	PAG Condition	n
Scopolamine group		
Vehicle	vehicle	7
Vehicle	morphine (2.5 ug)	7
Scopolamine (0.5 ug)	morphine (2.5 ug)	7
Scopolamine (5.0 ug)	morphine (2.5 ug)	7
Scopolamine (5.0 ug)	vehicle	6
Pirenzepine group		
Vehicle	vehicle	6
Vehicle	morphine (2.5 ug)	6
Pireniepine (0.O5ug)	morphine (2.5 ug)	6
Pirenzepine (0.5 ug)	morphine (2.5 ug)	6
Pireniepine (5.0 ug)	morphine (2.5 ug)	6
Pirenzepine (5.0 ug)	vehicle	6
Methoctramtrie group		
Vehicle	vehicle	11
Vehicle	morphine (2.5 ug)	6
Methoctramine (0.5 ug)	morphine (2.5 ug)	6
Methoctramine (2.5 ug)	morphine (2.5 ug)	6
Methoctramine (2.5 ug)	vehicle	5
Mecamylamine group		
Vehicle	vehicle	8
Vehicle	morphine (2.5 ug)	8
Mecamylamine (0.01 ug)	morphine (2.5 ug)	6
Mecamylamine (0.) ug)	morphine (2.5 ug)	7
Mecamylamine (1.0 ug)	morphine (2.5 ug)	8
Mecamylamine (1.0 ug)	vehicle	6
Control group		
Vehicle	vehicle	11
Vehicle	morphine (2.5 ug)	11
Scopolamine (5.0 ug)	morphine (2.5 ug)	6
Pirenzepine (5.0 ug)	morphine (2.5 ug)	5
Methoctramine (2.5 ug)	morphine (2.5 ug)	5
Mecamylamine (1.0 ug)	morphine (2.5 ug)	5

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

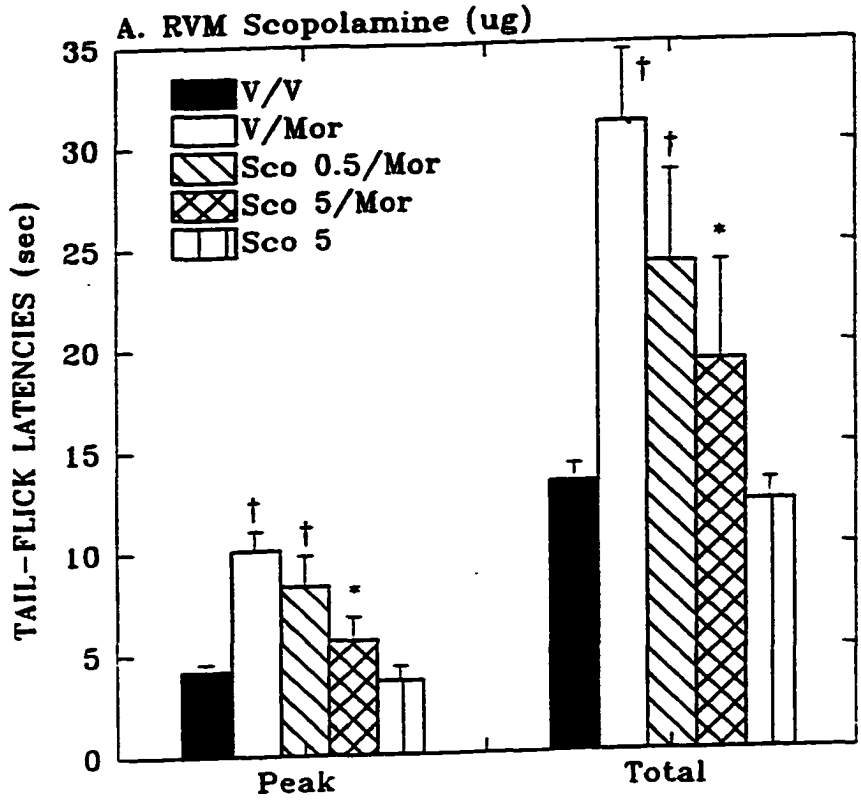
summarizes the treatments, doses, and sample sizes used to assess the antagonist effects of either scopolamine, muscarinic, pirenzepine (M_1), methoctramine (M_2), or mecamylamine (nicotinic) administered into the RVM upon morphine antinociception elicited from the vlPAG. The last section of Table 3 summarizes treatment of control rats with medullary placements lateral and/or dorsal to the RVM. Animals receiving particular antagonist doses were matched on the basis of their basal nociceptive latencies and thresholds as well as the degree of mesencephalic morphine antinociception. This injection regimen used counterbalancing procedures so that particular antagonist/agonist dose combinations were spaced equally across the five weekly conditions. It should be noted that a similar protocol was used in analgesic synergy studies between the vlPAG and RVM (Rossi, Pasternak, and Bodnar, 1993; Rossi, Pasternak, and Bodnar, 1994), and the analgesic efficacy of particular doses of opioid agonists including morphine did not differ as a function of repeated injections using this interval.

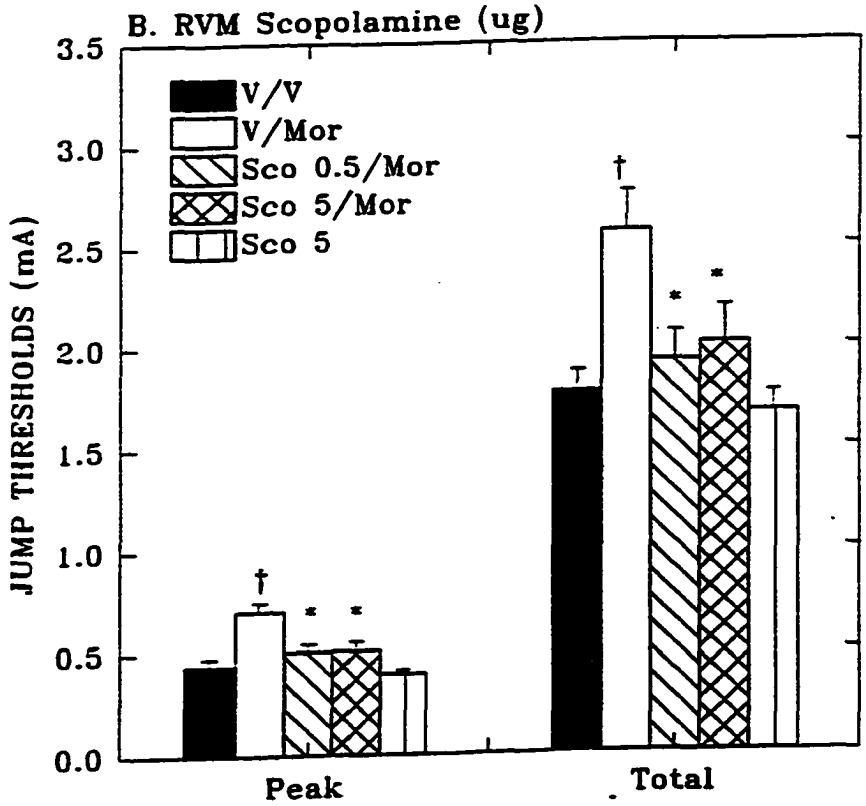
C. Results.

1. RVM Scopolamine and Mesencephalic Morphine Antinociception: Significant differences were observed for peak ($F_{4, 24} = 10.33$, $p < 0001$, and total ($F = 6.87$. $p < 0.0001$) effects on the tail-flick test, and for peak ($F = 21.87$, $p < 0.0001$) and total ($F = 16.92$. $p < 0.0001$) effects on the jump test. Morphine (2.5 μg) in the vlPAG significantly increased peak and total latencies and thresholds (Figure 5). RVM microinjections of scopolamine significantly and dose-dependently reduced mesencephalic morphine antinociception for both peak (5 μg, 76%) and total (5 μg, 67%) effects on the tail-flick test (Figure 5, upper panel) and for both peak (0.5 ug. 76%; 5 μg, 74%) and total (0.5 μg,

Figure 5 EXPERIMENT 2: RVM Scopolamine and Mesencephalic Morphine Antinociception. Alterations in tail flick latencies (top panel) and jump test thresholds (lower panel) in rats injected with 2.5 ug morphine in the vlPAG and scopolamine (0.5, 5.0 ug) in the RVM..

Figure 5





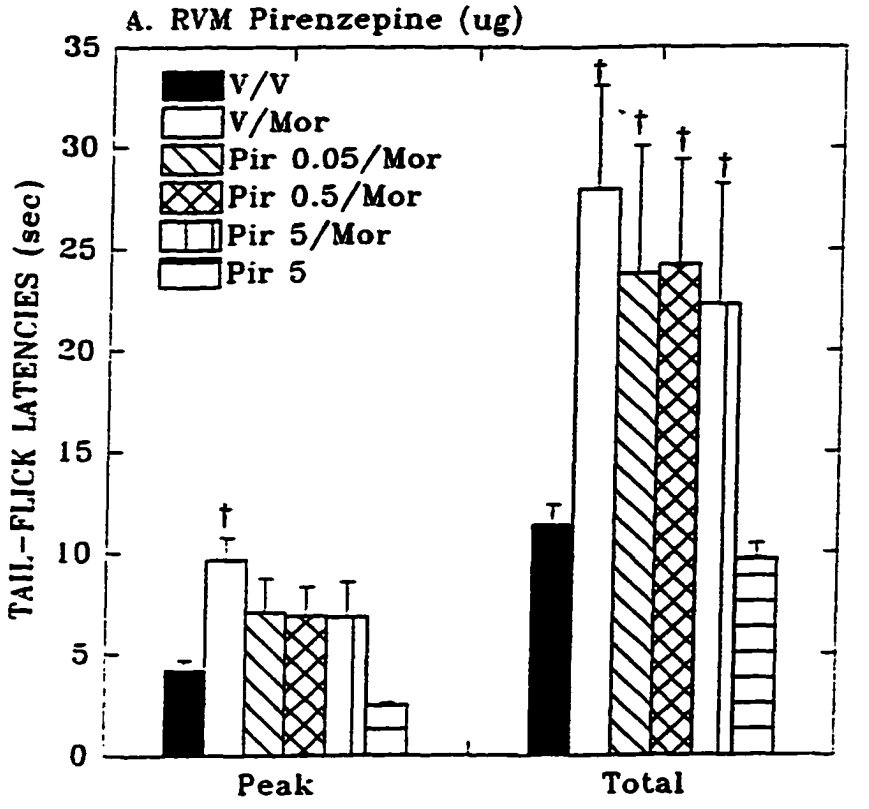
82%; 5 μg, 71%) effects on the jump test (Fig. 5B). In contrast, RVM microinjections of scopolamine failed to alter baseline latencies or thresholds.

2. RVM Pirenzepine and Mesencephalic Morphine Antinociception: Significant differences were observed for peak ($F_{5,25}$ =5.73, $p < 0.001$. and total ($F = 5.28$, $p < 0.0019$) effects on the tail-flick test and for peak ($F = 7.16$, $p < 0.0003$) and total ($F = 7.40$, $p < 0.0002$) effects on the jump test. Morphine in the vlPAG significantly increased peak and total latencies and thresholds (Figure 6). Although RVM microinjections of pirenzepine failed to significantly alter mesencephalic morphine antinociception for peak and total effects on the tail-flick test (Figure 6, upper panel), pirenzepine significantly reduced mesencephalic morphine antinociception for total (0.05 μg, 41%; 5ug, 39%), but not for peak effects on the jump test (Fig. 6B). Although RVM microinjections of pirenzepine decreased baseline latencies and increased baseline thresholds, these effects did not achieve statistical significance.

3. RVM Methoctramine and Mesencephalic Morphine Antinociception: Significant differences were observed for peak ($F_{4,40} = 121.05$, $p<0.0001$) and total ($F = 186.34$, $p<0.0001$) effects on the tail-flick test, and for peak ($F=77.79$, $p<0.0001$) and total ($F=107.63$, $p<0.001$) effects on the jump test. Morphine in the vlPAG significantly increased peak and total latencies and thresholds (Figure 7). Although RVM injections of methoctramine failed to alter mesencephalic morphine antinociception for peak and total effects on the tail-flick test (Fig. 7A), methoctramine significantly reduced mesencephalic morphine antinociception for peak (0.5 μg, 45%; 2.5 μg, 45%) and total (0.5 μg, 54%; 2.5 pg, 51%) effects on the jump test (Figure 7, lower panel). RVM

Figure 6 **EXPERIMENT 2: RVM Pirenzepine and Mesencephalic Morphine Antinociception. Alterations in tail flick latencies (top panel) and jump test thresholds (lower panel) in rats injected with 2.5 ug morphine in the vlPAG and pirenzepine (0.05, 0.5, 5.0 ug) in the RVM..**

Figure 6



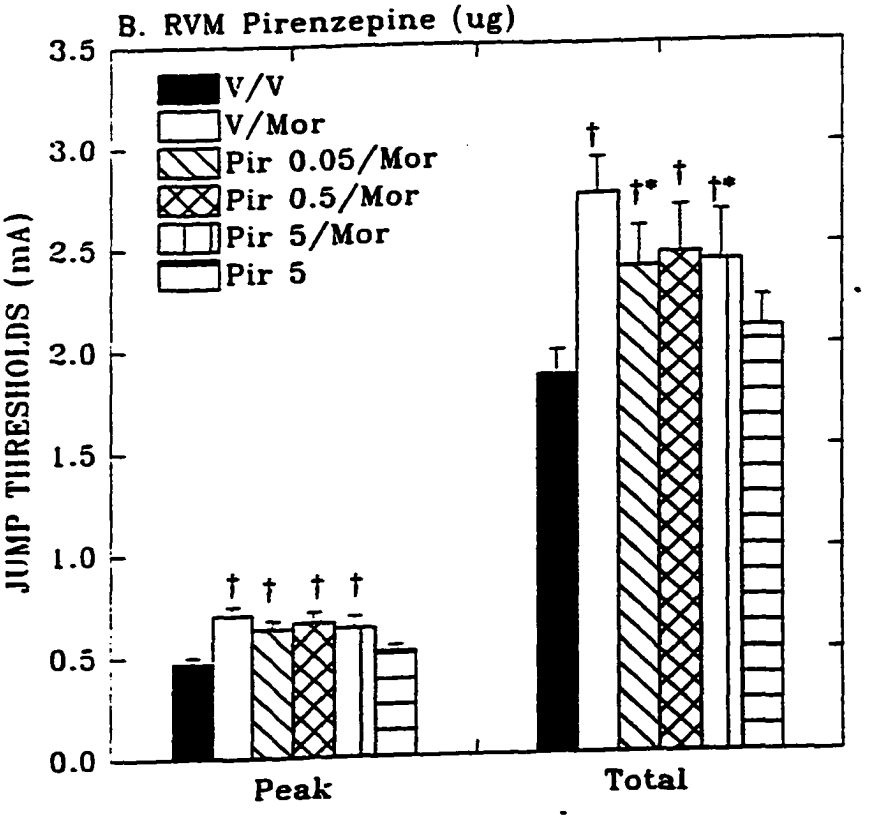
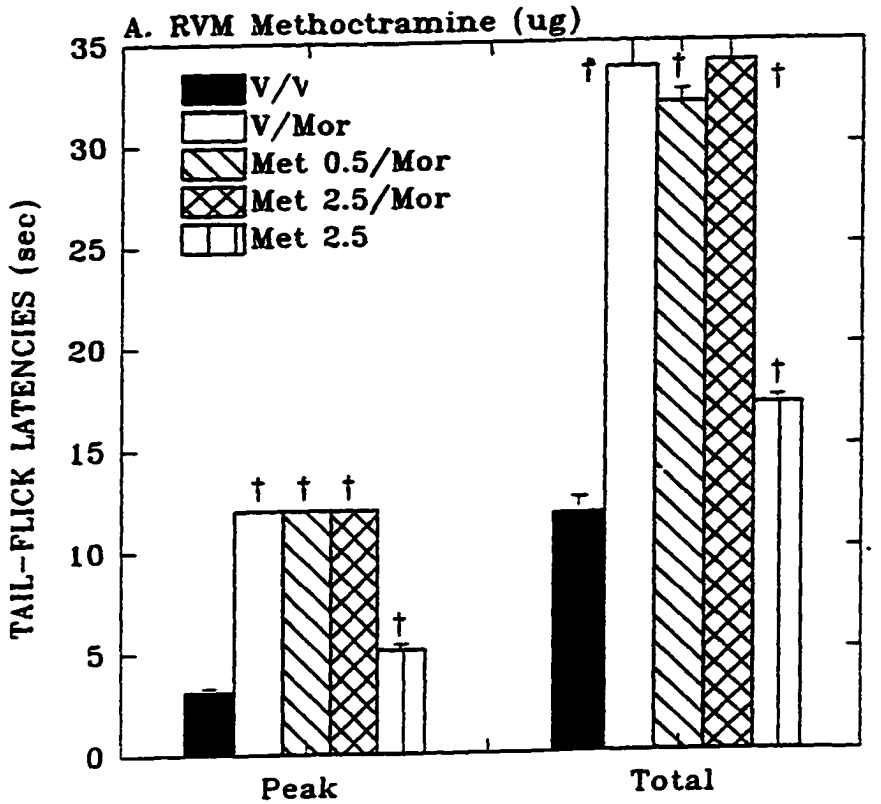
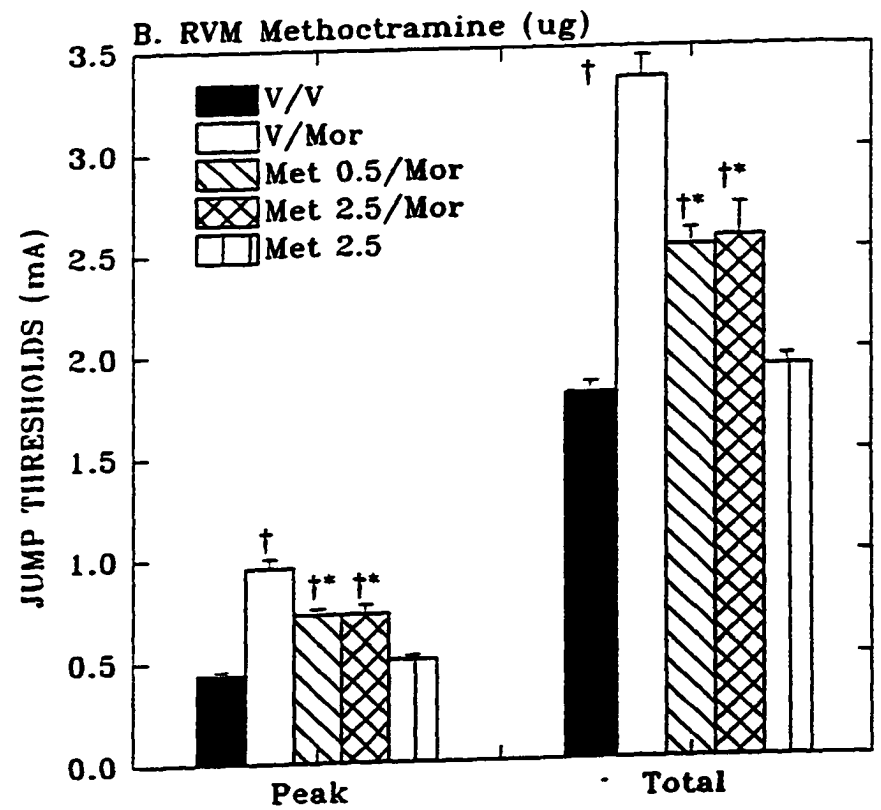


Figure 7 EXPERIMENT 2: RVM Methoctramine and Mesencephalic Morphine Antinociception. Alterations in tail flick latencies (top panel) and jump test thresholds (lower panel) in rats injected with 2.5 ug morphine in the vlPAG and methoctramine (0.5, 2.5 ug) in the RVM.

Figure 7





microinjections of methoctramine significantly increased baseline peak (62%) and total (30%) tail-flick latencies, but failed to affect baseline jump thresholds (Figure 7).

4. RVM Mecamylamine and Mesencephalic Morphine Antinociception: Significant differences were observed for peak ($F_{5, 35} = 44.37$, $p < 0.0001$, and total ($F = 34.37$, $p < 0.0001$) effects on the tail-flick test, and for peak ($F = 25.98$, $p < 0.0001$) and total ($F = 17.18$, $p < 0.0001$) effects on the jump test. Morphine in the vlPAG significantly increased peak and total latencies and thresholds (Figure 8). RVM microinjections of mecamylamine significantly and dose-dependently reduced mesencephalic morphine antinociception for peak (0.1 μg, 58%; 1 μg, 88%) and total (0.01 μg, 24%; 0.1 μg, 53%; 1 μg, 85%) effects on the tail-flick test (Figure 8, upper panel), and for peak (0.1 μg, 57%; 1 μg, 76%) and total (0.01 μg, 30%; 0.1 μg, 67%; 1 μg, 65%) effects on the jump test (Figure 8, lower panel). RVM microinjections of mecamylamine failed to alter baseline latencies and thresholds.

5. Mesencephalic Morphine Antinociception and Cholinergic Antagonists in Lateral Medullary Placements: Significant differences were observed for peak ($F_{5, 50} = 67.73$, $p < 0.0001$), and total ($F = 58.48$, $p < 0.0001$) effects on the tail-flick test, and for peak ($F = 34.17$, $p < 0.0001$) and total ($F = 29.96$, $p < 0.0001$) effects on the jump test. Morphine in the vlPAG significantly increased peak and total latencies and thresholds (Table 4). Misplaced medullary microinjections of scopolamine failed to alter mesencephalic morphine antinociception on either the tail-flick or jump test. Although misplaced medullary microinjections of pirenzepine failed to alter mesencephalic morphine antinociception on the tail-flick test, pirenzepine significantly reduced

Figure 8 EXPERIMENT 2: RVM Mecamylamine and Mesencephalic Morphine Antinociception. Alterations in tail flick latencies (top panel) and jump test thresholds (lower panel) in rats injected with 2.5 ug morphine in the vlPAG and mecamylamine (0.01, 0.1, 1.0 ug) in the RVM.

Figure 8

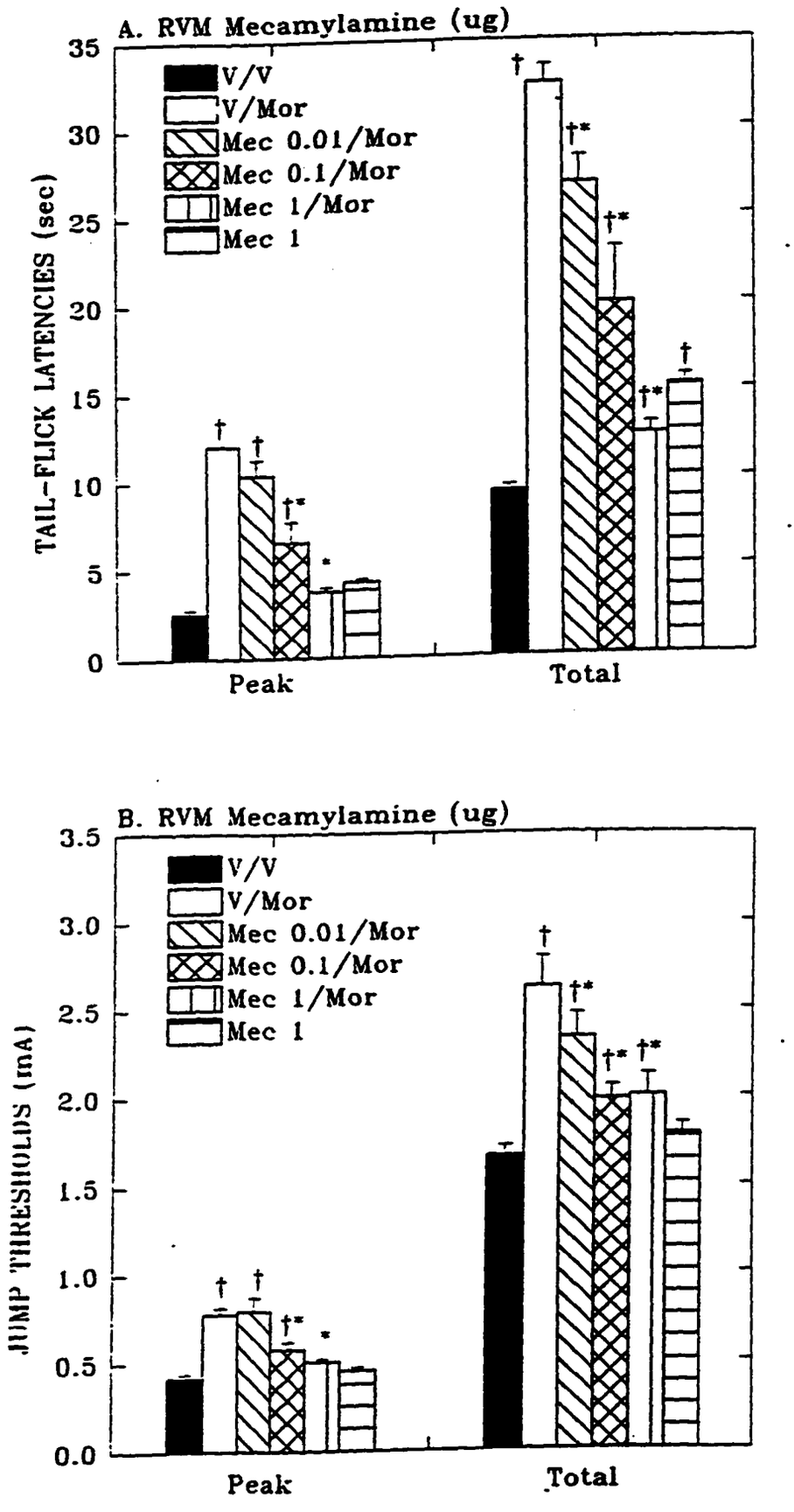


TABLE 4

EXPERIMENT 2: Alterations in Mesencephalic Morphine Analgesia by Preeatment of Either Muscarinic or Nictinic Cholinergic Antagonists in Medullary Sites Lateral and Dorsal to the Rostral Ventromedial Medulla

	Tail Flick Latencies (s)		Jump Thresholds (mA)	
Condition	Peak Effects	Total Effects	Peak Effects	Total Effects
Veh/Veh	2.78±0.14	10.96±0.47	0.389±0.013	1.558±0.069
Veh/MOr	11.21±0.53*	30.91±1.63*	0.918±0.049*	2.944±0.014*
Sco/Mor	9.98±0.72*	31.14±1.78*	0.839±0.040*	2.698±0.999*
Pir/Mor	12.00±0.00*	36.45±0.56*+	0.813±0.027*	2.517±0.080*+
Niet/NIor	6.75±0.59*+	19.76±1.48*+	0.667±0.039*+	2.209±0.115*+
Mec/Mor	12.00±0.00*	24.98±0.39*+	0.690±0.029*+	2.084±0.047*+

Values are mean ± SEM. Veh, vehicle; Mor, morphine (2.5 ug); Sco, scopolamine (5 ug); Pir, pirenzepine (5 ug); Met, methoctramine (2.5 ug); Mec, mecamylamine (1 ug). The first injection was administered into the misplaced medullary placement and the second injection was administered into the periaqueductal gray.
*Significant difference relative to corresponding Veh/Veh (Tukey comparison, $p < 0.05$).
+Significant difference relative to corresponding VehIMor (Tukey comparison, $p < 0.05$).

mesencephalic morphine antinociception for total (31%) effects on the jump test. Misplaced medullary microinjections of methoctramine significantly reduced mesencephalic morphine antinociception for peak (53%) and total (56%) effects on the tail-flick test, and for peak (47%) and total (53%) effects on the jump test. Misplaced medullary microinjections of mecamylamine significantly reduced mesencephalic morphine antinociception for total (30%), but not peak effects on the tail-flick test, and for peak (43%) and total (62%) effects on the jump test.

D. Discussion.

1. Muscarinic Cholinergic Antagonism and Mesencephalic Morphine Antinociception: The present study confirmed that muscarinic cholinergic receptors in the RVM mediate in part the analgesic responses induced by morphine microinjected into the vlPAG. The muscarinic cholinergic antagonist, scopolamine, microinjected into the RVM produced potent dose-dependent reductions in mesencephalic morphine antinociception on both the tail-flick and jump tests both in terms of the peak magnitude of effects and the overall total duration of effects. The actions of this general muscarinic cholinergic antagonist in the RVM in blocking the expression of mesencephalic morphine antinociception were specific because scopolamine in the RVM failed to alter baseline latencies and thresholds. This rules out the possibility of a hyperalgesic effect of this antagonist that could nonspecifically counteract morphine's antinociceptive actions in the vlPAG. Further, muscarinic cholinergic antagonism with scopolamine of mesenchephalic morphine antinociception was localized to the RVM because scopolamine microinjected into misplaced medullary cannulae dorsal and/or lateral to the

RVM failed to alter this antinociceptive response. Therefore, scopolamine's selective pharmacological and anatomical actions in the RVM in reducing mesencephalic morphine antinociception are similar in pattern and in magnitude to the ability of serotonergic (5-HT_1, 5-HT_2, 5-HT_3), opioid (general, μ, δ), and excitatory amino acid (NMDA) antagonists to produce the same effects in this site (Kiefel, Cooper, and Bodnar, 1992a; Kiefel, Cooper, and Bodnar, 1992b; Kiefel, Rossi, and Bodnar, 1993; Spinella, Cooper, and Bodnar, 1996).

That muscarinic cholinergic antagonism in the RVM is active in mediating supraspinal opioid antinociceptive processes is consistent with the ability of the muscarinic cholinergic agonist, carbachol, to increase nociceptive thresholds following RVM administration (Brodie and Proudfit, 1984; Brodie and Proudfit, 1986; Klamt and Prado, 1991), and indicates a supraspinal site of action. This effect is in addition to the inhibition of morphine antinociception observed following i.t. administration of muscarinic cholinergic antagonists (Chiang and Zhuo, 1989; Fang and Proudfit, 1996). Iontophoretic application of acetylcholine excites a majority of RVM cells (Behbehani, 1982), indicating a functional excitatory role for muscarinic receptors (Kobayashi, Palkovits, Hruska, Rothschild, and Yamamura, 1978). Further, the muscarinic agonist, (+)-cis-methyldioxolane, produced antinociception in the RVM that was blocked by either the selective M_1 antagonist, pirenzepine, or the irreversible muscarinic receptor antagonist, 4-diphenylacetoxy-N-[2-chloroethyl]-piperidine mustard (Iwamoto and Marion, 1994). Moreover, this muscarinic-mediated antinociception in the RVM is mediated by nitric oxide because it is blocked by the nitric oxide synthase inhibitor, L-

N^G-nitroarginine in the RVM (Iwamoto and Marion, 1994). Thus, these data suggest that muscarinic cholinergic receptors in the RVM responsible for supraspinal muscarinic antinociception also are necessary for the full expression of mesencephalic morphine antinociception.

2. M_1 and M_2 Cholinergic Antagonism and Mesencephalic Morphine Antinociception. To ascertain the relative contribution of M_1 and M_2 receptor subtypes present in the RVM (Cortes and Palacios, 1986,Nonaka and Morojii, 1984; Spencer, Horvath, and Traber, 1986; Wamsley, Zarbin, Birdsall, and Kuhar, 1980) in the mediation of mesencephalic morphine antinociception, the present study examined effects of RVM administration of M_1-selective (pirenzepine) and M_2-selective (methoctramine) antagonists. The results were not as convincing, consistent, or specific as the scopolamine effects. First, RVM administration of pirenzepine only significantly decreased the duration (total effect) of mesencephalic morphine antinociception on the jump test, with the size of the effect considerably smaller for pirenzepine (39-41%) relative to scopolamine (71-82%). Second, RVM pirenzepine failed to significantly alter either the magnitude (peak effect) of mesencephalic morphine antinociception on the jump test or the magnitude and duration of mesencephalic morphine antinociception on the tail-flick test; these measures were markedly altered by scopolamine treatment. These and subsequent test-specific effects may reflect the relative participation of this antagonist in differentially mediating each pain test at different levels of the neuraxis. Whereas responses to the tail-flick test (D'Amour and Smith, 1941) are mediated at the level of the spinal cord based upon anatomical (Grossman, Basbaum, and Fields, 1982)

and behavioral (see reviews: Bodnar, 1986; Terman, Shavit, Lewis, Cannon, and Liebeskind, 1994) evidence, responses to the jump test are mediated by supraspinal mechanisms. Alternatively, these effects may merely reflect potency differences, which would be rectified by administration of higher antagonist doses that are affected by solubility and volume limitations. Third, the limited positive actions of pirenzepine on the duration of mesencephalic morphine antinociception was not anatomically limited to the RVM because microinjections of pirenzepine into sites lateral and/or dorsal to the RVM produced the same magnitude of reductions (31%) as RVM treatment (39-41%). Thus, these limited M_1 receptor-mediated actions upon mesencephalic morphine antinociception appear to involve a number of medullary structures, and distinguish this opioid antinociceptive response from antinociception elicited by RVM administration of muscarinic agonists, which are eliminated by pirenzepine pretreatment in the RVM (Iwamoto and Marion, 1994).

Iwamoto and Marion (1994) have previously shown that the selective M_2 receptor antagonist, methoctramine, produces antinociception following RVM administration, and cotreatment with muscarinic agonists produces an additive antinociceptive effect on the hot plate and tail-flick tests. The present study confirmed that RVM methoctramine effectively increased baseline tail-flick latencies, but did not alter thresholds on a nonthermal nociceptive response to shock. Iwamoto and Marion (1994) have hypothesized that the basal antinociception elicited by methoctramine is due to the presynaptic autoreceptor mechanisms of M_2 receptors in which stimulation of this receptor would decrease acetylcholine release, and blockade of the receptor would

enhance acetylcholine release. Interestingly, the M_2 antagonist, methoctramine, significantly reduces mesencephalic morphine antinociception on that nociceptive test, the jump test, where it fails to elicit basal antinociception. Methoctramine fails to generally alter mesencephalic morphine antinociception on that nociceptive test, the tail-flick test, where it produces basal increases in latencies. However, the reductions in mesencephalic morphine antinociception on the jump test by methoctramine are not limited to the RVM because the magnitudes of decreased within the RVM (45-51%) and dorsal and/or lateral to the RVM (47-53%) were quite comparable.

The data indicating that general muscarinic cholinergic antagonism with scopolamine produced reductions in mesencephalic morphine antinociception only when microinjected into the RVM suggest actions at both postsynaptic (M_1) and presynaptic (M_2) receptor sites. However, the relative inability of individual M_1 and M_2 receptor antagonists to produce comparable magnitudes of effect, anatomical specificity of effect, and generalizability of effect across nociceptive tests indicates that muscarinic mediation of mesencephalic morphine antinociception in the medulla is not simple and specific. Several important caveats need to be addressed. The first issue is the injection volume (1 ul) used to administer antagonists into the medulla. This volume was necessitated by the limited solubility of the antagonists in a vehicle solution that would not produce effects itself. Given that both RVM and lateral medullary guide cannulae penetrated through the fourth ventricle, it is conceivable that this volume of injectate could travel up the injector track after removal of the internal cannula, and thereby be dispersed to neighboring structures. Procedural steps were performed to minimize this possibility by waiting at

least 30 sec after completion of the injection to allow the injectate to be absorbed by tissue. The second issue concerns the temporal relationship between antagonist and agonist administration. Because all of the muscarinic antagonists are reversible, a fixed (20 min) interval between medullary antagonist administration and morphine administration into the vlPAG was employed. This interval is comparable to the i.t. pretreatment of muscarinic antagonists either 10 min prior to RVM administration of nicotine (Iwamoto and Marion, 1993a) or 15 min prior to i.t. administration of muscarinic agonists (Iwamoto and Marion, 1993b). It is conceivable that this interval allowed the antagonists to diffuse from the original injection site. Because M_1 and M_2 antagonists produced similar magnitudes of reduction in both RVM and more lateral medullary placements, it is difficult to address this issue. This possibility was not apparent in our earlier studies with serotonergic, opioid, and NMDA receptor antagonists because RVM, but not lateral medullary placements, elicited reductions in mesencephalic morphine antinociception (Kiefel et al., 1992a; Kiefel et al., 1992b; Kiefel et al., 1993; Spinella et al., 1996). The third issue concerns potential differential diffusion of the antagonist within the ventral medulla either from or to the active site. Whether scopolamine, pirenzepine, or methoctramine possess differential diffusion capabilities is not known, and again the present study was designed based upon the previous clear-cut anatomical specificity observed for serotonergic, opioid, and NMDA antagonists. A fourth and final issue is the potential use of a combined M_1 (pirenzepine) and M_2 (methoctramine) antagonist cocktail, which would presumably act identically to scopolamine in producing robust and site-specific effects. Problems with solubility,

potential pharmacokinetic effects, and precise choices of appropriate pairs of antagonist doses preclude definitive applicability of this procedure. Biochemical assays using such a cocktail have not been performed. Therefore, with these caveats, it appears that muscarinic receptor antagonism in ventral medullary placements reduce mesencephalic morphine antinociception with the greatest magnitudes of decreases noted when both M_1 and M_2 receptors are blocked with scopolamine treatment.

3. Nicotinic Cholinergic Receptor Antagonism and Mesencephalic Morphine Antinociception. The present data indicate that the nicotinic cholinergic antagonist, mecamylamine, microinjected into the RVM significantly and dose-dependently reduced mesencephalic morphine antinociception on the tail-flick test for peak (58-88%) and total (53-85%) effects and on the jump test for peak (57-76%) and total (65-67%) effects. These effects were observed in the absence of mecamylamine effects upon baseline latencies and thresholds. However, if mecamylamine was administered into sites lateral and/ or dorsal to the RVM, it significantly reduced mesencephalic morphine antinociception, particularly on the jump test. Therefore, the specificity of nicotinic cholinergic receptor antagonism upon mesencephalic morphine antinociception cannot be limited to the RVM as observed for general muscarinic antagonism in the present study, and observed for serotonergic, opioid, and NMDA receptor antagonism in previous studies (Kiefel, Cooper, and Bodnar, 1992a, 1992b; Kiefel, Rossi, and Bodnar, 1993; Spinella, Cooper, and Bodnar, 1996).

Nicotinic receptor agonists have been shown to produce a potent antinociceptive response following microinjection into the RVM as well as the PPTg (Iwamoto, 1989,

1991). This supraspinal antinociceptive response appears to depend upon the integrity of M_1 cholinergic receptors in these nuclei because pirenzepine pretreatment significantly reduces this antinociceptive response. Nicotinic-induced antinociception appears to depend upon a pathway that extends from the PPTg to the RVM (Iwamoto, 1991), and further from the RVM to the spinal cord using adrenergic, serotonergic, and cholinergic, but not opioid, spinal synapses (Iwamoto, 1993). Similarly, systemic nicotine antinociception is also mediated by spinal adrenergic, serotonergic, and cholinergic receptors (Rogers and Iwamoto, 1993), suggesting that systemic cholinergic stimulation activates either a supraspinal cholinergic system and/or a spinal component. Cholinergic muscarinic agonists also produce spinally mediated antinociception following i.t. administration (Gillberg, Gordh, Hartvig, Jansson, Peterson, and Post, 1989; Gillberg, Hartvig, Gordh, Sottile, Jansson, Archer, and Post, 1990; Iwamoto and Marion, 1986; Iwamoto and Marion, 1993b; Smith, Yang, Nha, and Buccafusco, 1989; Taylor, Yaksh, and Richelson, 1983; Yaksh, Dirksen, and Harty, 1985; Zhou and Gebhart, 1991), and, as indicated previously, spinal muscarinic cholinergic receptors mediate mesenchephalic morphine antinociception in thermal nociceptive tests (Fang and Proudfit, 1996). These latter authors found comparable inhibitory effects using a general (atropine) muscarinic cholinergic antagonist in the spinal cord as the present study found with a general (scopolamine) muscarinic cholinergic antagonist in the RVM. However, the receptor subtype mediation of i.t.-effective antagonists and the site specificity have not been determined.

Previous studies have determined that the RVM is a critical site for the integrity

of mesencephalic morphine antinociception in that serotonergic (5-HT_1, 5-HT_2, 5-HT_3) and opioid (general, μ, δ) microinjected into this nucleus significantly reduce the magnitude and duration of mesencephalic morphine antinociception (Kiefel, Cooper, and Bodnar, 1992a; Kiefel, Cooper, and Bodnar, 1992b; Kiefel, Rossi, and Bodnar, 1993; Spinella, Cooper, and Bodnar, 1996). Further, whereas neurotensin antagonists administered into the RVM significantly enhance mesencephalic morphine antinociception, neurotensin in the RVM significantly reduces mesencephalic morphine antinociception (Urban and Smith, 1993, 1994). Two other strict criteria have been used to determine the selectivity and specificity of these effects. First, the given antagonist should not alter basal latencies and thresholds following RVM administration to eliminate possible nonselective countervailing hyperalgesic effects. Second, the given antagonist should be effective in reducing mesencephalic morphine antinociception only when microinjected into the RVM, not into sites dorsal and/or lateral to this structure. The present study indicated that only the general muscarinic cholinergic antagonist, scopolamine, satisfied all of these criteria and produced consistent dose-dependent reductions in mesencephalic morphine antinociception both in terms of magnitude and duration on the tail-flick and jump tests. The M_1 receptor antagonist, pirenzepine, effective in reducing muscarinic-mediated antinociception in the RVM (Iwamoto, 1989, 1991), produced minor effects upon mesencephalic morphine antinociception that were observed either within or surrounding the RVM. The antinociceptive actions of the M_2 receptor antagonist, methoctramme, in the RVM were confirmed (Marion and Iwamoto, 1994), and again any reductions in mesencephalic morphine antinociception were

observed in sites that were either within or surrounding the RVM. Finally, nicotinic receptor antagonism with mecamylamine displayed the same nonselective effects in that sites within or surrounding the RVM reduced mesencephalic morphine antinociception following this antagonist.

V. EXPERIMENT 3

A. Introduction

It appears that some opioid agonists, including morphine and BEND, employ different anatomical and neurochemical pathways in exerting their supraspinal antinociceptive effects. Ventricular morphine and BEND antinociception are dissociated in that intrathecal naloxone blocked BEND, but not morphine antinociception (Tseng and Fujimoto, 1985). Immunoreactive spinal met-enkephalin is released following ventricular and hypothalamic administration of BEND, but not morphine (Tseng et al., 1985; Tseng and Wang, 1992). Both antibodies to met-enkephalin and spinal antagonists block BEND, but not morphine antinociception (Suh and Tseng, 1990a; Tseng and Suh, 1989). Ventricular morphine and BEND fail to develop antinociceptive cross-tolerance (Suh and Tseng, 1990b), and are differentially altered by pentobarbital anesthesia (Tseng and Tang, 1992). Morphine and BEND only display additive antinociceptive effects following ventricular and intrathecal administration (Roerig et al., 1988). Within the vlPAG, barbiturate anesthesia respectively reduces and enhances morphine and BEND antinociception (Smith et al., 1992a). Whereas BEND antinociception in the vlPAG is dependent upon a spinal opioid component, morphine antinociception in the vlPAG is dependent upon spinal adrenergic and serotonergic receptors (Suh et al., 1988; Suh et al., 1989; Tseng and Collins, 1991; Tseng and Tang, 1990). Although naltrexone and the mu-selective antagonist, CTOP both blocked morphine and BEND antinociception elicited from the vlPAG, the slopes of the dose-inhibition curves were not parallel (Smith et al., 1992b; Monroe et al., 1996), suggesting involvement of distinct receptor subpopulations.

Since mediation of supraspinal BEND antinociception by the RVM has not been examined, the present study examined whether either competitive (AP7) or noncompetitive (MK-801) NMDA anatagonists, or muscarinic (scopolamine) or nicotinic (mecamylamine) cholinergic antagonists administered into the RVM at effective or higher doses than those reducing mesencephalic morphine antinociception (Spinella et al., 1996; Spinella et al., 1997), would alter BEND antinociception elicited from the vlPAG as measured by the tail-flick test, a measure of thermal reactivity (D□Amour and Smith, 1941), and the jump test, a measure of nociceptive reactivity to shock (Evans, 1961). To insure that BEND antinociception elicited from the vlPAG was mediated through an opioid synapse, the ability of naltrexone pretreatment in the vlPAG to block this antinociceptive response was evaluated.

B. Protocols.

Rats in the first experiment were exposed to a maximum of four microinjection conditions at weekly intervals: a) RVM vehicle (1 mul)/vlPAG vehicle (1 mul) (n=14), b) RVM vehicle (1 mul)/vlPAG BEND (15 μg) (n=14), RVM MK-801 doses of c) 3 ug (n=5) or d) 10 ug (n=8) paired with vlPAG BEND (15 μg), RVM AP7 doses of e) 3 ug (n=5) or f) 10 ug (n=5) paired with vlPAG BEND (15 μg), g) RVM scopolamine (5 ug)/vlPAG BEND (15 μg) (n=5), and h) RVM mecamylamine (1 ug)/vlPAG BEND (15 μg) (n=5). Higher doses of scopolamine (10 μg) and mecamylamine (3 μg) were tested in the RVM of control animals and induced seizures. Therefore, these higher doses were not employed in the present study. Rats in each of the antagonist conditions were matched on the basis of latency and threshold scores attained under vehicle conditions and agonist

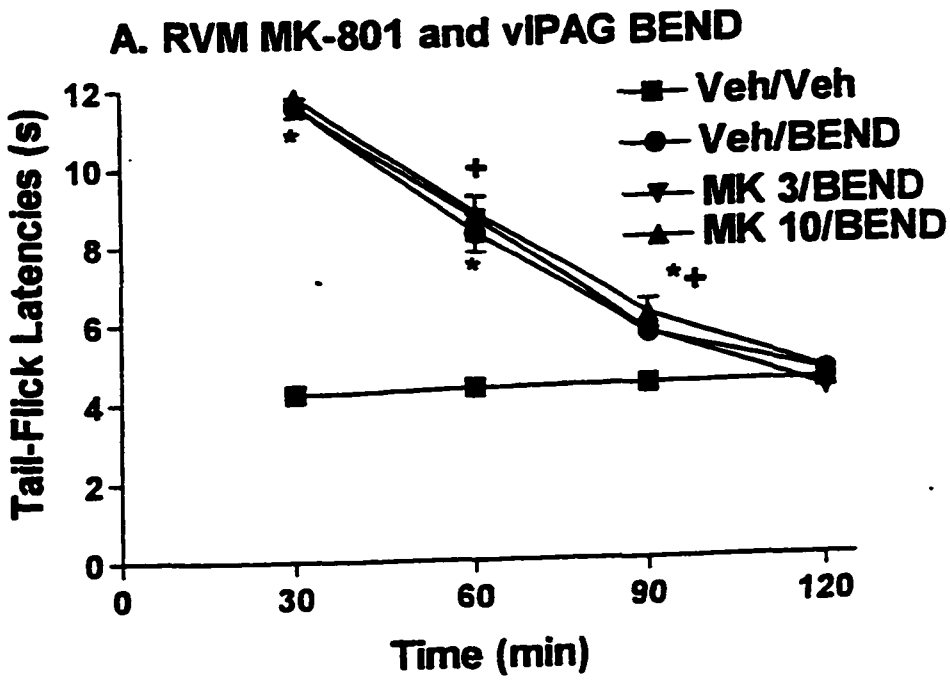
conditions. Latencies and thresholds were determined at 30, 60, 90 and 120 min following the second microinjection of each pair. Five rats in the second experiment were exposed to the following conditions in the vlPAG at weekly intervals: a) vehicle (1 ul)/vehicle (1 ul), b) vehicle (1 ul)/ BEND (15 μg), and naltrexone at doses of c) 1 ug or d) 20 ug paired with BEND (15 μg). Latencies and thresholds were again determined at 30, 60, 90 and 120 min following the second microinjection of each pair.

C. Results.

1. NMDA RVM Antagonists and vlPAG BEND Antinociception: Significant differences were observed among conditions (tail-flick: $F(7,91)= 101.65$, $p<.0001$; jump: $F= 70.53$, $p<.0001$), across test times (tail-flick: $F_{3,39}= 2148.24$, $p<.0001$; jump: $F= 2226.76$, $p<.0001$) and for the interaction between conditions and times (tail-flick: $F_{21,273}= 64.97$, $p<.0001$; jump: $F= 54.82$, $p<.0001$). BEND significantly increased latencies and thresholds for up to 90 min following vlPAG microinjection relative to vehicle/vehicle treatment. The lower (3 μg) dose of the noncompetitive NMDA antagonist, MK-801 in the RVM failed to affect BEND antinociception in the vlPAG on either test (Figures 9A and 9B); this dose completely eliminated vlPAG morphine antinociception (Spinella et al., 1996). As the RVM MK-801 dose was raised to 10 μg, BEND antinociception in the vlPAG was transiently (30 min) and minimally (9%) decreased on the tail-flick, but not jump tests. The competitive NMDA antagonist, AP7 at a 3 ug dose in the RVM transiently (30 min) and minimally (11%) decreased BEND antinociception on the jump, but not the tail-flick tests (Figures 9C and 9D), a dose which eliminated morphine antinociception in the vlPAG (24). A higher (10 μg) dose of AP7 in

Figure 9 EXPERIMENT 3: RVM MK801 and RVM AP7 and and Mesencephalic BEND Antinociception. Alterations in (a) tail flick latencies and (b) jump test thresholds in rats injected with 15 ug BEND in the vlPAG and MK-801 (3, 10 ug) in the RVM.. Alterations in (c) tail flick latencies and (d) jump test thresholds in rats injected with 15 ug BEND in the vlPAG and AP7 (3, 10 ug) in the RVM.

Figure 9



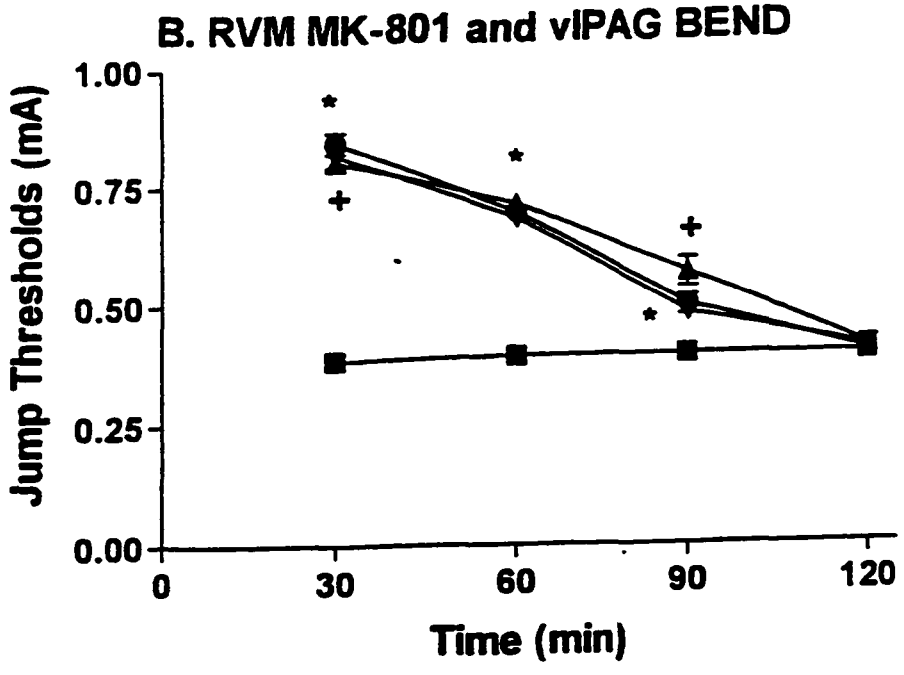
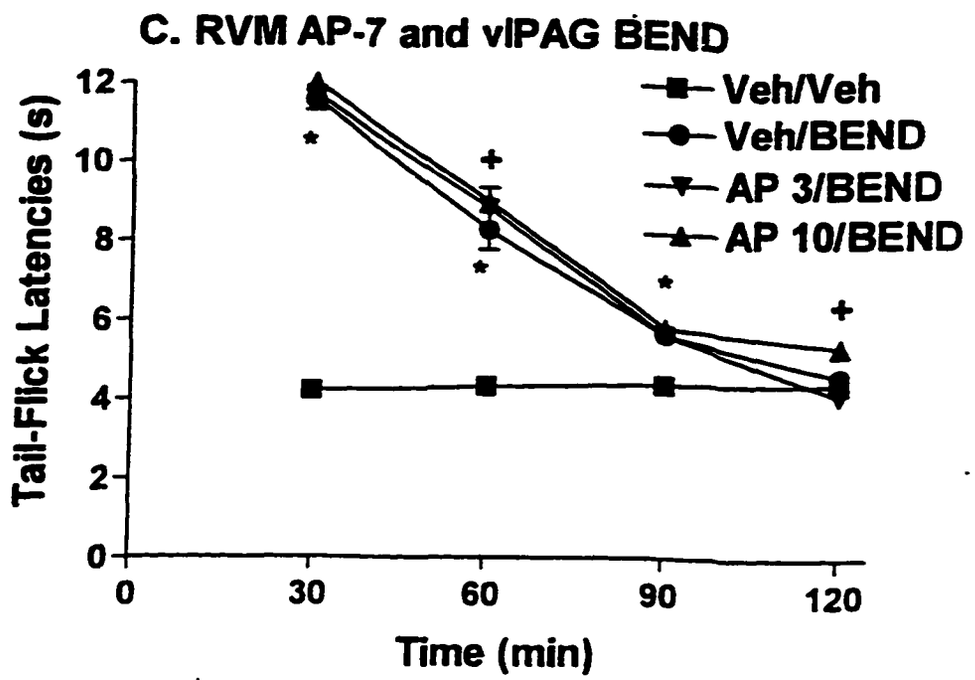


Figure 9



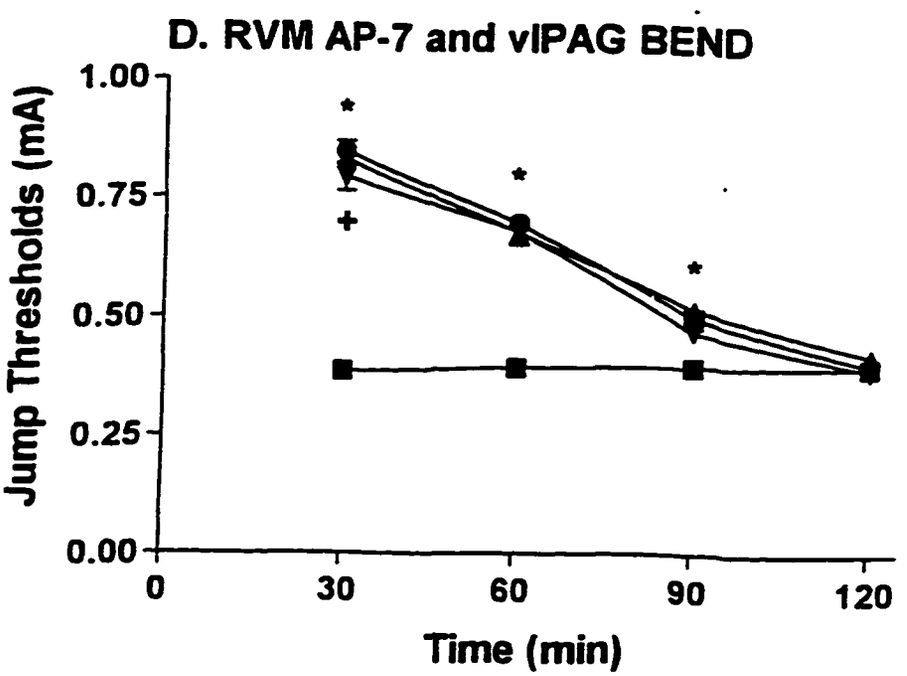


TABLE 5

EXPERIMENT 3: SUMMARY OF EXPERIMENTAL GROUPS

RVM condition	PAG condition	n
A. MK-801 group		
Vehicle	Vehicle	10
Vehicle	BEND (15 ug)	10
MK-801 (3.0 ug)	BEND (15 ug)	5
MK-801 (10 ug)	BEND (15 ug)	10
B. AP7 group		
Vehicle	Vehicle	6
Vehicle	BEND (15 ug)	6
AP7 (3 ug)	BEND (15 ug)	5
AP7 (10 ug)	BEND (15 ug)	6
C. Scopolamine group		
Vehicle	Vehicle	5
Vehicle	BEND (15 ug)	5
Scopolamine (5 ug)	BEND (15 ug)	5
D. Mecamylamine group		
Vehicle	Vehicle	5
Vehicle	BEND (15 ug)	5
Mecamylamine (1 ug)	BEND (15 ug)	5
PAG condition	PAG condition	n
E. Naltrexone group		
Vehicle	Vehicle	6
Vehicle	BEND (15 ug)	6
Naltrexone (1 ug)	BEND (15 ug)	6
Naltrexone (20 ug)	BEND (15 ug)	6

TABLE 6

EXPERIMENT 3: Alterations in Mesencephalic BEND Analgesia by Preeatment of Either EAA (MK-801 or AP7) or Cholinergic (Scopolamine, Mecamylamine) Antagonists in Medullary Sites Lateral and Dorsal to the Rostral Ventromedial Medulla.

Veh -Vehicle; BEND - beta-endorphin (15 ug); MK-801a (3 ug); MK-801b(10 ug ug); AP7a (3 ug); AP7b (10 ug). Scop - scoplolamine (1 ug), Mec - mecamylamine (1 ug). The first injection was administered into the medullary placement and the second injection was administered into the periaqueductal gray.

Condition	Post-injection time (min)			
	30	60	90	120
Tail-flick latencies (sec)				
Veh/Veh	4.20	4.33	4.39	4.41
Veh/BEND	11.57*	8.25*	5.64*	4.62
MK801a/BEND	11.58*	8.64*	5.70*	4.18
MK801b/BEND	11.81*+	8.77*+	6.14*+	4.66
AP7a/BEND	11.68*	8.82*	5.66*	4.12
AP7b/BEND	12.00*	8.97*+	5.83*	5.37*+
Scop/BEND	12.00*	7.35*+	5.50*	4.25
Mec/BEND	12.00*	7.87*	6.24*+	4.02+
Jump Thresholds (mA)				
Veh/Veh	.385	.393	.396	.395
Veh/BEND	.845*	.695*	.498*	.397
MK801a/BEND	.816*	.682*	.481*	.407
MK801b/BEND	.804*+	.713*	.567*+	.408
AP7a/BEND	.790*+	.687*	.470*	.388
AP7b/BEND	.829*	.675*	.517*	.419
Scop/BEND	.822*	.698*	.503*	.413
Mec/BEND	.836*	.703*	.488*	.399

* Significant difference relative to corresponding Veh/Veb (Dunnett comparison, $p < 0.05$).

\+ Significant difference relative to corresponding Veh/Mor (Dunn comparison, $p < 0.05$).

TABLE 7

EXPERIMENT 3: Alterations in Mesencephalic BEND Analgesia by Preeatment With Naltrexone in the vlPAG.

Veh -Vehicle; BEND - beta-endorphin (15 ug); NTXa - Naltrexone (1 ug); NTXb - Naltrexone (20 ug). The first and second injections were administered into the vlPAG.

Condition	Post-injection time (mm)			
	30	60	90	120
Tail-flick latencies (sec)				
Veh/Veh	5.14	5.19	5.18	4.93
Veh/BEND	11.52*	9.75*	6.09	5.26
NTXa/BEND	8.05*+	7.63*+	5.63	4.87
NTXb/BEND	4.09+	5.19+	5.11+	4.66
Jump Thresholds (mA)				
Veh/Veh	.420	.422	.425	.421
Veh/BEND	.782*	.678*	.508*	.422
NTXa/BEND	.612*+	.563*+	.505*	.415
NTXb/BEND	.423+	.418+	.428+	.434

* Significant difference relative to corresponding Veh/Veb (Dunnett comparison, $p < 0.05$).

\+ Significant difference relative to corresponding Veh/Mor (Dunn comparison, $p < 0.05$).

the RVM produced small increases after 60 and 120 min in the magnitude of BEND antinociception in the vlPAG on the jump, but not tail-flick tests.

2. Cholinergic RVM Antagonists and vlPAG BEND Antinociception: The muscarinic cholinergic antagonist, scopolamine, in the RVM failed to alter the magnitude of BEND antinociception on the jump test, and produced transient (30 min) and small (23%) reductions on the tail-flick test (Figures 10A and 10B) following a 5 ug dose which eliminated morphine antinociception in the vlPAG on both tests (Spinella et al., 1997). Similarly, the nicotinic cholinergic antagonist, mecamylamine in the RVM failed to alter the magnitude of BEND antinociception in the vlPAG on the jump test, and produced transient (90 min) increases in BEND antinociception in the vlPAG on the tail-flick test (Figures 10A and 10B) following a 1 ug dose which markedly reduced morphine antinociception in the vlPAG on both tests.

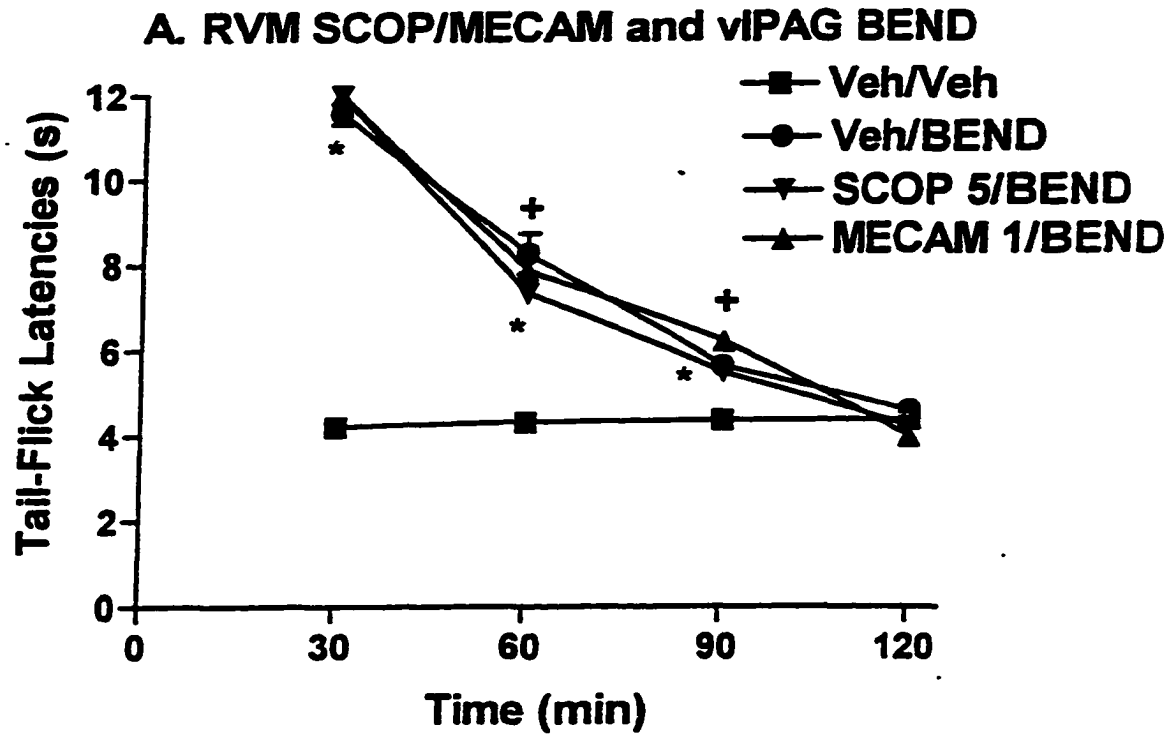
3. vlPAG Naltrexone and vlPAG BEND Antinociception: Significant differences were observed among conditions (tail-flick: $F_{3,16}$= 30.30, p<.0001; jump: F= 8.61, p<.001), across test times (tail-flick: F(3,48)= 47.38, p<.0001; jump: F= 47.82, p<.0001) and for the interaction between conditions and times (tail-flick: $F_{9,48}$= 16.81, p<.0001; jump: F= 22.10, p<.0001). BEND in the vlPAG significantly increased latencies and thresholds for up to 60 and 90 min respectively relative to vehicle/vehicle treatment. Naltrexone pretreatment in the vlPAG significantly and dose-dependently reduced BEND antinociception in the vlPAG with the 1 ug dose reducing latencies (50%) and thresholds (41%), and the 20 ug dose eliminating antinociceptive effects on both tests (Figures 11A and 11B).

D. Discussion.

The present study demonstrated that neither NMDA or cholinergic receptor subtype antagonists microinjected into the RVM markedly altered BEND antinociception elicited from the vlPAG. These data are clearly dissociated from the elimination of morphine antinociception elicited from the vlPAG following RVM pretreatment of either NMDA or cholinergic receptor subtype antagonists (Spinella et al., 1996; Spinella et al., 1997).

Figure 10 EXPERIMENT 3: RVM Scopolamine & RVM Mecamylamine and Mesencephalic BEND Antinociception. Alterations in tail flick latencies and jump test thresholds in rats injected with 15 ug BEND in the vlPAG with (a) scopolamine (5 ug) or (b) mecamylamine (1 ug) in the RVM.

Figure 10



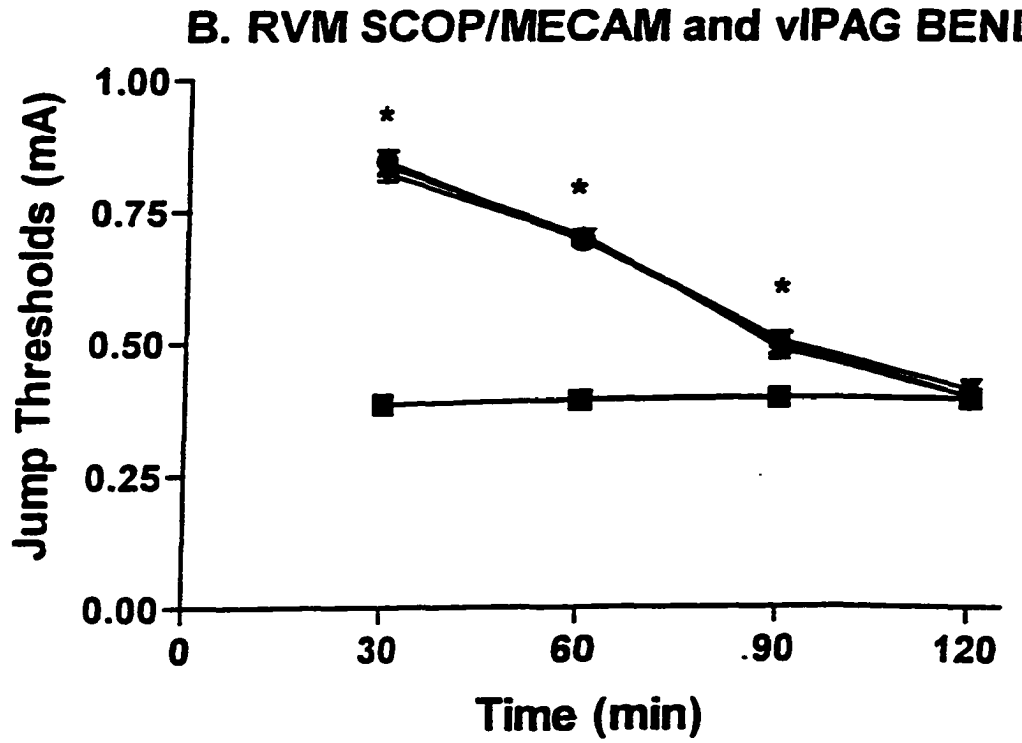
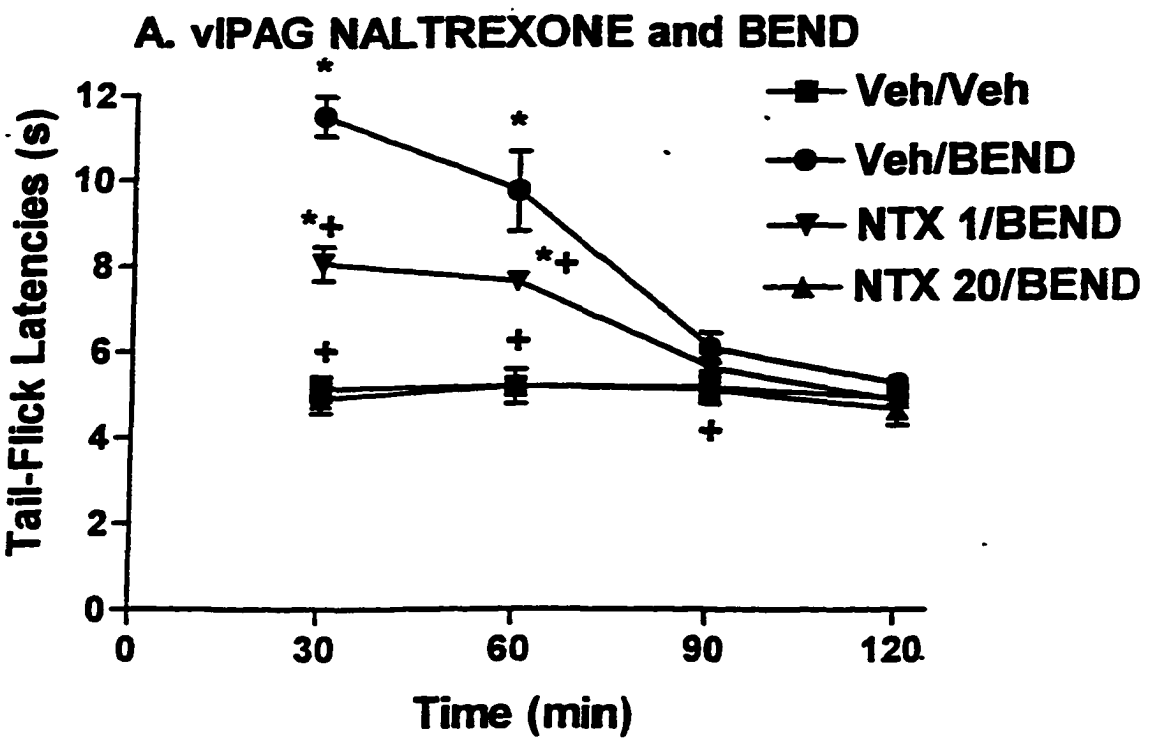
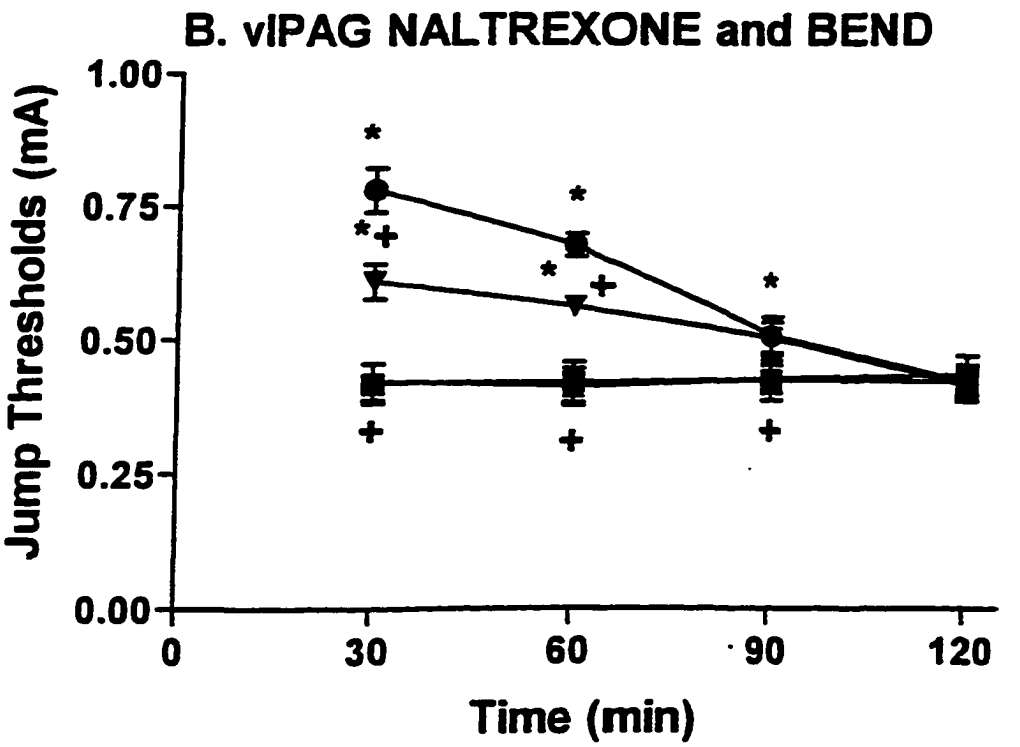


Figure 11 EXPERIMENT 3: Mesencephalic Natrexone and Mesencephalic BEND. Alterations in (a) tail flick latencies and (b) jump test thresholds in rats injected with naltrexone (1, 20 ug) followed by BEND (15 ug) in the vlPAG.

Figure 11





Mesencephalic morphine antinociception was eliminated by a 3 ug dose of MK-801 in the RVM, and was markedly reduced by RVM MK-801 doses of 0.03 and 0.3 ug (Spinella et al., 1996). Similarly, RVM AP7 doses between 0.1 and 1 ug markedly reduced mesencephalic morphine antinociception. These noncompetitive and competitive NMDA-mediated effects in the RVM were specific for three reasons. First, the kainate/AMPA antagonist, CNQX failed to exert meaningful effects in the RVM upon mesencephalic morphine antinociception. Second, administration of NMDA antagonists into sites dorsal and lateral to the RVM failed to alter mesencephalic morphine antinociception. Finally, the small hyperalgesic actions of RVM MK-801 upon baseline nociceptive measures could not account for its marked effects upon mesencephalic morphine antinociception. The maximum degree of reductions of mesencephalic BEND antinociception by RVM microinjections of either MK-801 or AP7 at 3 and 10 ug doses was 9-11% at peak (30 min) effects, and was followed by subsequent small increases in the magnitude of mesencephalic BEND antinociception on both tests. Thus, it does not appear that NMDA receptors in the RVM mediate the potent antinociceptive actions of BEND elicited from the vlPAG.

The present experiment showed that BEND antinociception elicited from the vlPAG was unaffected by RVM pretreatment with a nicotinic antagonist, and displayed only a minimal (23%) and transient (30 min) reduction on the tail-flick, but not jump test following muscarinic antagonism in the RVM. Thus, it does not appear that cholinergic receptors in the RVM mediate the potent antinociceptive actions of BEND elicited from the vlPAG.

The present data extend the findings of important dissociations between morphine and BEND antinociception elicited from the vlPAG, including differential responses to barbiturate anesthesia (Smith et al., 1992a), and differential mediation of vlPAG morphine antinociception by spinal adrenergic and serotonergic receptors and of BEND antinociception by spinal opioid receptors (Suh et al., 1988; Suh et al., 1989; Tseng and Collins, 1991; Tseng and Tang, 1990). Separate subpopulations of mu-opioid receptors appear to mediate morphine and BEND antinociception elicited from the vlPAG given the non-parallel slopes of the dose-inhibition curves elicited by vlPAG administration of naltrexone and the mu-opioid antagonist, CTOP (Smith et al., 1992b; Monroe et al., 1996). The opioid actions of BEND in mediating antinociception elicited from the vlPAG were confirmed in the present study by the dose-dependent inhibition by vlPAG naltrexone. It should be noted that BEND and morphine antinociception exhibit similar profiles of reductions when these agonists are microinjected into the amygdala and naltrexone is administered into the vlPAG (Pavlovic et al., 1996; Tershner and Helmstetter, 1995). Further, microinjections of the δ_2 opioid antagonist, naltrindole isothiocyanate and, to a lesser degree the μ opioid antagonists, B-funaltrexamine and CTOP, into the vlPAG significantly reduce antinociception induced by either BEND or morphine administered into the amygdala. However, synergy studies (Pavlovic and Bodnar, 1998) between the amygdala and the vlPAG reveal differences in antinociceptive interactions especially when BEND is administered into the latter structure. Thus, co-administration of morphine into both the amygdala and vlPAG produces a profound synergistic antinociceptive interaction regardless of which site received the fixed dose of

morphine and which site received the variable morphine dose. An identical pattern of synergistic antinociceptive interactions was observed following co-administration of BEND into both the amygdala and vlPAG. Co-administration of BEND (1 μg) into the amygdala and morphine (1 μg) into the vlPAG produced a potent interaction, but co-administration of morphine into the amygdala and BEND into the vlPAG failed to produce any interactive effects, indicating differential forms of antinociceptive processing in the vlPAG between the two opioid agonists.

The inability of NMDA or cholinergic receptor antagonists in the RVM to reduce BEND antinociception elicited from the vlPAG leaves open two possibilities in delineating descending pathways subserving supraspinal BEND antinociception. The first possibility is that BEND antinociception elicited from the vlPAG still utilizes the RVM as an effective pathway, but that neither NMDA nor cholinergic synapses play a role in these effects. Serotonergic ($5HT_2$ and $5HT_3$), opioid (mu, δ) and neurotensin systems in the RVM may be involved in mediating BEND antinociception elicited from the vlPAG given their antagonists□ abilities to either reduce (serotonin and opioids) or enhance (neurotensin) morphine antinociception elicited from the same structure (Kiefel et al., 1992a; Kiefel et al., 1992b; Kiefel et al., 1993; Urban and Smith, 1993; Urban and Smith, 1994). Further, $GABA_A$ and $GABA_B$ receptors in the RVM also modulate antinociceptive processes (McGowan and Hammond, 1993a; McGowan and Hammond, 1993b; Heinricher et al., 1991; Proudfit, 1980; Urban and Smith, 1994b). Antagonist studies altering these receptor systems in the RVM should be evaluated for effects upon morphine and BEND antinociception elicited from the vlPAG. Alternatively, one or more

of these and other receptors may be involved necessitating studies examining reversible inactivation of the RVM which reduces morphine antinociception (Proudfit, 1980; Urban and Smith, 1994b). The second possibility is that BEND antinociception elicited from the vlPAG utilizes a pathway(s) outside of the RVM through which its descending controls modulate spinal opioid release to produce pain-inhibitory effects (Tseng and Fujimoto, 1985; Tseng et al., 1985; Tseng and Wang, 1992; Suh and Tseng, 1990a; Tseng and Suh, 1989).

VI. GENERAL DISCUSSION

The findings in these experiments demonstrated dose- and time-responive effects. The blockade of antinociception created by microinjection of morphine and BEND into the vlPAG by cholinergic and NMDA antagonists was found to persist across the 120 minute span of testing. This time period which exceeds the length of significant antinociception created by either opioid agonist alone. Furthermore, these effects were dose-dependent. Injections of NMDA, muscarinic, and nicotinic antagonists dose-dependently reduced antinociception produced by administration of morphine into the vlPAG. The reduction of the magnitude of morphine's antinociceptive effect decreased with logarithmically smaller doses of antagonists. Relative differences were observed in the potencies of the antagonists at the doses studied.

The above experiments also demonstrated pharmacological specificity of the interactions between supraspinal opioid and non-opioid neurotransmitters mediating antinociception. The reduction of morphine antinociception produced by injection into the vlPAG was selectively and potently reduced by cholinergic antagonists working at muscarinic (scopolamine), nicotinic (mecamylamine), and EAA antagonists working at the NMDA (MK-801, AP7), but not non-NMDA receptors (CNQX). Further, these reductions in antinociception were specific to morphine antinociception, and not generalized to BEND antinociception. Although both opioids were believed to operate through the μ receptor, significant differences have been shown to exist between morphine and BEND antinociception at supraspinal and spinal levels, including differential sensitivity to barbiturates in the vlPAG, sensitivity to μ antagonists in the

vlPAG, and mediation by different neurotransmitters and receptors. Further, there is a lack of cross-tolerance and synergistic effects when morphine and BEND are co-administered. Thus, the systems by which morphine and BEND produce anagesia seem to be discrete to some degree. Congruent with this finding is the differential modulation of morphine and BEND antinociception by EAA and cholinergic antagonists. No effect was observed on BEND antinociception, even at doses of antagonist an order of magnitude higher than those which produced maximal reductions in morphine antinociception.

Specificity was also demonstrated in these studies on the anatomical level. Whereas injections of NMDA and ACh antagonists into the RVM produced a potent reduction of antinociception, injectections into regions lateral and dorsal to the RVM were ineffective in this respect. This further supports the antinociceptive pathway described by Basbaum and Fields (1978), where descending antinociceptive influence from the vlPAG can be halted by select antagonists in the RVM, but not surrounding areas.

The reduction of morphine antinociception by NMDA, muscarinic, and nicotinic antagonists was evident on both jump test and jump tests. This indicates that the effects are generalized to spinal as well as supraspinal levels.

Administration of the antagonist alone did not reduce nociceptive threshold below basal levels. Thus, the dose-dependent reduction of morphine antinociception by NMDA and ACh antagonists is due to specific pharmacological actions within a functional system, rather than a non-specific cancellation effect.

The differences obtained between the reduction by NMDA and ACh antagonists of morphine and BEND antinociception are not easily explained by existing studies. Although clear differences have been shown between the antinociception produced by supraspinally-administered BEND and morphine. The reason for this may be that BEND is acting potently at a receptor other than the μ receptor. However, such a receptor has not been demonstrated pharmacologically or genetically. A second possibility is that BEND may bind to a different part of the μ receptor than morphine, which may be altered in different splice variants or polymorphisms of the μ receptor. For example, ASODNs directed against exons 1 and 4 of MOR1 selectively prevented mu_1-mediated supraspinal morphine antinociception, while μ_2-mediated spinal antinociception was blocked by ASODNs directed against exon 4 (Rossi et al., 1995). M6G, on the other hand antinociception was only blocked by ASODN for exons 2 and 3. Thus, alternate splicing of the receptor gene can create pharmacological receptor subtypes and selectively alter the binding properties of the receptor for specific ligands. It is conceivable then, that the actions of BEND at the μ receptor may differ between its splice variants, which can have different differential anatomical distributions (as do the μ_1 and μ_2 receptors, even though they are coded for by the same gene.) Indeed, naturally-occuring single nucleotide polymorphisms of the human μ receptor have been identified in humans (Bond et al., 1998). Furthermore, these polymorphisms (found in exons 1 and 3) alter the binding affinity of the μ receptor for BEND, but not other endomorphin-1, another endogenous μ agonists.

In conclusion, the pharmacological and anatomical evidence presented here are

suggestive of discrete morphine- and BEND-activated antinociceptive systems. Although opioid systems are known to have some degree of convergence, there is also reason to believe that some separate, individual systems exist at some levels.

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IMAGE EVALUATION
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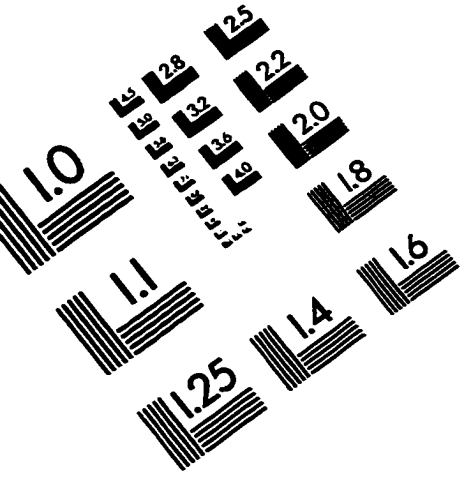

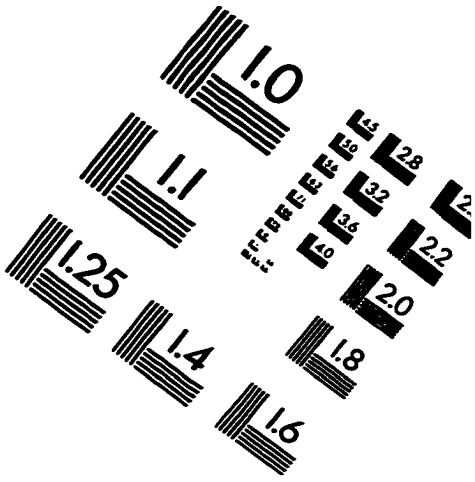

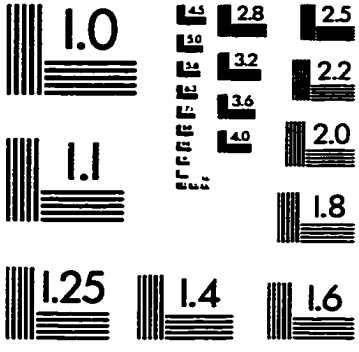

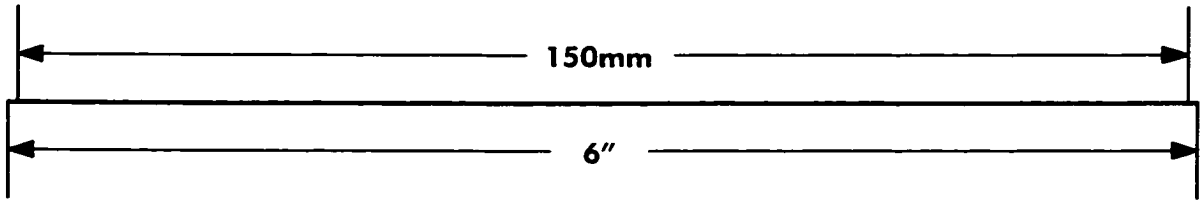

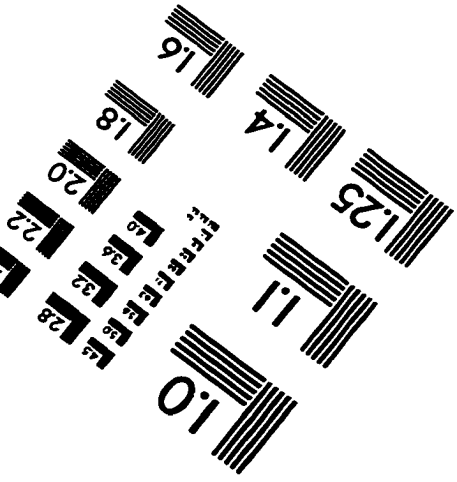

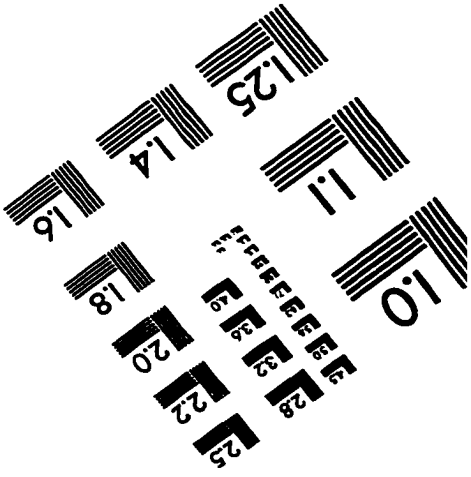

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