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Maturational changes in analgesia produced by glutamate and morphine stimulation of the periaqueductal gray in infant rats

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MATURATIONAL CHANGES IN ANALGESIA PRODUCED BY GLUTAMATE AND MORPHINE STIMULATION OF THE PERIAQUEDUCTAL GRAY IN INFANT RATS

by

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Abstract

Maturational changes in analgesia produced by glutamate and morphine stimulation of the periaqueductal grey in infant rats.

by

Leslie A. Tive

Advisor: Professor Gordon A. Barr

The periaqueductal grey matter (PAG) of the midbrain supports both opiate-induced analgesia (OA) and stimulation-produced analgesia (SPA) in adult animals and humans. However, topographical differences have been noted within the PAG that are related to the ability of this structure to support opiate and non-opiate forms of analgesia. In general, the dorsal aspect of the PAG mediates stimulation-produced analgesia more effectively, while the ventral aspect of the PAG mediates both opiate-induced and stimulation-produced analgesia. One way to distinguish the analgesia supported by dorsal and ventral PAG sites is to study the analgesia produced by stimulation of, or opiate administration to the PAG in developing animals. The aim of this thesis was to compare the postnatal development of the analgesia produced by glutamate and morphine administration to the dorsal and ventral PAG of the midbrain in the rat.

The first set of experiments examined the ontogeny of analgesia produced by glutamate or morphine microinjection into the dorsal and the ventral PAG. Pups aged 3, 10, and 14 days were implanted with cannulae aimed at either the dorsal or the ventral PAG. After recovery, each pup was given an injection of one dose of morphine (vehicle, 2 μg or 6μg) or glutamate (vehicle, 60 mM or 180 mM) and tested for analgesia against noxious thermal and mechanical stimuli. Pups were then euthanized and implant sites within the PAG were verified. Morphine inducing analgesia at 3 and 10 days of age only when administered to the ventral part of the PAG and only against the thermal noxious stimulus. Conversely, glutamate-produced analgesia was seen at 3 and 10 days of age only when glutamate was given to the dorsal aspect of the PAG and only against the mechanical noxious stimulus. In 14 day old pups, both drugs produced analgesia against both types of noxious stimuli regardless of their site of administration within the PAG. These results indicated that the morphine-induced analgesia and glutamate-produced analgesia mediated by the PAG can be differentiated developmentally. The PAG can be functionally subdivided into a dorsal and a ventral region, with each region supporting either glutamate-produced or morphine-

induced analgesia early in development and both regions supporting both types of analgesia in the more mature animal.

The second set of experiments investigated the ability of the opiate antagonist naloxone to reverse the analgesia supported by dorsal and ventral PAG sites. When glutamate or morphine was administered to the ventral PAG systemic naloxone attenuated the resultant analgesia. When glutamate or morphine was given to the dorsal PAG, systemic naloxone did not attenuate the analgesia produced by these compounds. These data indicate that analgesia supported by the ventral PAG is opioid in nature, whereas the analgesia supported by the ventral PAG is not opioid in nature.

One possible interpretation of these results was that morphine-induced analgesia and stimulation-produced analgesia supported by the PAG are differentially modulated by descending bulbospinal monoamine systems. To test this hypothesis, the third group of experiments compared the roles of spinal serotonin (5-HT) and norepinephrine (NE) in the production of morphine-induced and glutamate-produced analgesia mediated by the dorsal and ventral PAG in developing rats. Fourteen day old rat pups were implanted with cannulae aimed at either the dorsal or the ventral PAG and with intrathecal catheters. Glutamate (180 mM) was administered to the dorsal or ventral PAG followed immediately by an intrathecal (i.t.) injection of either the α–noradrenergic antagonist phentolamine (15 or 30 μg/4 μl) or the serotonergic antagonist methysergide (15 or 30 μg/4 μl). Morphine (6 μg) was administered to the ventral PAG only followed by i.t. administration of either phentolamine or methysergide. Analgesia was assessed against noxious thermal and mechanical stimuli. The results showed that when glutamate or morphine was given to the ventral aspect of the PAG, methysergide (15 μg/4 μl) attenuated analgesia against the thermal stimulus to a significantly greater degree than the mechanical stimulus while phentolamine (15 μg/4 μl) attenuated analgesia against the mechanical stimulus to a significantly greater degree. When glutamate was given to the dorsal PAG, analgesia against both stimulus types was attenuated to the same degree by intrathecal methysergide. However, when glutamate was given to dorsal PAG sites, intrathecal phentolamine attenuated the analgesia against the mechanical stimulus more effectively. This stimulus effect was abolished by high doses (30 μg/4 μl) of intrathecal phentolamine or methysergide in conjunction with intraPAG glutamate or morphine.

These findings suggest that glutamate and morphine administered to the PAG activate a common descending bulbospinal system that serves to modulate nociception. In addition, this study showed that there are distinct differences in the way that spinal monoamines modulate antinociception produced by dorsal and ventral PAG sites. Moreover, this study confirmed the hypothesis put forth by other groups that spinal serotonergic systems exert a stronger modulatory effect on thermal noxious stimuli than on mechanical noxious stimuli while the reverse is true for spinal noradrenergic systems.

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This entire work is dedicated to the memory of my mother, Diana Rafalow Grossman, who was with me in spirit during every moment of this endeavor and who taught me as a child that winners never quit and quitters never win.

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General Introduction

In 1969, Reynolds observed that electrical stimulation of the mesencephalic central grey produced sufficient analgesia to perform a laporatomy. Since then, many studies have shown that that electrical stimulation of medial brainstem sites will produce behavioral analgesia in rats (Balagura and Ralph, 1973; McCreery and Bloedel, 1975; Mayer et al., 1971; Soper, 1976), cats (Liebeskind et al, 1973; Melzack and Melinkoff, 1974; Oliveras et al., 1974), rhesus monkeys (Goodman and Holcombe, 1976; Ruda et al, 1976), and humans (Gybels et al., 1976, Hosobuchi et al., 1977, Richardson and Akil, 1977). In all of these studies, the magnitude of the analgesic effect varied with the intensity, frequency and duration of the stimulation pulses. Moderate stimulation was shown to totally eliminate overt withdrawal responses to heat and destructive tissue pinch (Liebeskind et al., 1973; Melzack and Melinkoff, 1974).

The PAG supports both SPA and OA in several species of animal and humans. Behavioral analgesia was seen against a variety of noxious stimuli following electrical stimulation of the PAG, stimulation of the PAG using the excitatory neurotransmitter glutamate (GPA) and stimulation of the receptors in the PAG with the opiate morphine. The OA, GPA and SPA supported by the PAG share some common mechanisms of action, yet the precise nature of the neuroanatomical and functional relationship between these types of analgesia is not clear. For example, the SPA supported by the ventral PAG is cross-tolerant with morphine and antagonized by the opiate antagonist naloxone, while the SPA supported by the dorsal PAG is not (Akil and Liebeskind, 1975; Akil et al., 1976; Cannon et al., 1982; Morgan and Liebeskind, 1987). This study examined the relationship between GPA and OA elicited by the PAG by comparing the development of both types of analgesia in rat pups. Developmental comparisons were also made between the OA and GPA supported by dorsal and ventral sites within the PAG.

Morphine-Induced Analgesia

Morphine-induced analgesia is mediated by μ receptors in the PAG of the midbrain

Morphine is thought to induce analgesia by stimulating μ opiate receptors in both the PAG and in the dorsal horn of the spinal cord. Numerous studies have shown that microinjections of morphine into medial brainstem structures extending from the rostral

portions of the fourth ventricle surrounding the cerebral aqueduct into the regions around the caudal aspect of the third ventricle will produce behavioral analgesia (Criswell, 1976; Herz, et al,1970; Jaquet,1974; Lewis and Gebhart, 1977; Mayer and Murfin,1976; Pert and Yaksh, 1974; Sharpe, et al, 1974; Tsou and Jang, 1964). These studies yielded consistent results in a wide variety of species including the mouse (Criswell, 1976), the rabbit (Herz, 1970; Tsou and Jang, 1964) the rat (Jaquet, 1974; Lewis and Gebhart, 1977; Mayer and Murfin, 1976) and primates (Pert and Yaksh, 1974) using different types of standard tests to assess analgesia including the the tail flick test, the hot-plate test, the flinch jump test and the pinch test. Regardless of the assay used, morphine injection to the PAG area induced measurable analgesia. It is likely that systemically administered morphine acts, at least in part, on receptors in the PAG to induce analgesia, as cross-tolerance develops between systemic and intraPAG administrations of morphine.

Morphine-induced analgesia is also mediated by cells in the dorsal horn of the spinal cord

Intrathecal injections of various opiates including morphine induce antinociception that is dose-dependent, stereospecific, and reversed by the opiate antagonist naloxone (Yaksh, 1981,1982; Yaksh and Rudy, 1976 and 1977). Opioid receptors were localized in the dorsal horn of the spinal cord using the autoradiographic technique. Radiolabelled naloxone was used to demonstrate the presence of opiate receptors in the superficial layers (lamina I and II) of the dorsal horn. These receptors were identified pharmacologically as the μ and δ type opiate receptors. Partial destruction of the small diameter primary pain afferents in the dorsal horn (the Aδ and C fibers) caused a reduction in the number of both types of opiate receptors. Morphine is thought to act directly on opioid receptors in the dorsal horn of the spinal cord that interact with the primary pain afferents. The function of morphine at the spinal level is probably to inhibit the activity of these pain afferents and thereby block noxious impulses from entering the dorsal horn of the spinal cord.Several areas of the brain such as the amygdala, the corpus striatum and the hypothalamus also contain large number of μ opiate binding sites (Kuhar et al, 1973). However, morphine injection into these areas did not result in analgesia, indicating that opiate binding alone is not a sufficient condition for inducing analgesia

Morphine administered supraspinally causes changes in the responses of dorsal horn neurons

There is considerable evidence that morphine binding in the PAG induces analgesia by inhibiting the activity of spinal nociceptive neurons. The first suggestion of this came from the work of Irwin et al in 1951. They found that the antinociceptive effects of systemically administered morphine on the tail flick reflex were attenuated in animals with transsected spinal cords. They concluded that morphine activates a supraspinal antinociceptive system that descends to the spinal cord and inhibits the transmission of nociceptive information in a manner similar to that of intrathecally administered morphine. Subsequently, many reports have substantiated this theory. Bennett and Mayer (1976) showed that morphine microinjections into the PAG inhibited the responses of spinal cord neurons to nociceptive stimuli. This data suggested that morphine administered supraspinally may induce analgesia by way of a descending inhibitory pathway. They also demonstrated that dorsal horn neurons that were unresponsive to nociceptive stimuli were unaffected by morphine microinjection, indicating that this inhibition is relatively specific. Destruction of the dorsolateral funiculus (DLF) of the spinal cord partially antagonized the antinociceptive effect of systemically administered morphine (Basbaum et al,1977; Hayes, et al,1978; Price et al, 1976) and almost totally abolished the antinociceptive effect of morphine injected into the PAG (Murfin et al, 1976) in tests measuring both spinally mediated nociceptive reflexes and nociceptive responses that require supraspinal organization. The fact that DLF lesions had a more profound antagonistic effect on morphine administered to the PAG than on systemically administered morphine indicates that peripherally administered morphine acts both on supraspinal and spinal opiate receptors, while morphine administered to the PAG probably acts exclusively on supraspinal opiate receptors.

Collectively, these studies indicate that morphine induces analgesia in two distinct ways. One way is by binding to opiate receptors in the dorsal horn and inhibiting the responses of primary pain afferents entering the spinal cord, thereby blocking the processing of painful stimuli. The other way is by stimulating μ receptors in the PAG and activating a descending spinopetal system. This system enters the the spinal cord through the DLF and inhibits the responding of primary pain afferents to noxious stimuli entering the spinal cord. Direct projections from the PAG to the spinal cord do exist (Kuypers and Maisky, 1975, 1977; Kniesley, 1978; Leichnetz et al.,1978) although they are relatively sparse (Ruda, 1975; Kuypers, 1975). It is thought that stimulating the PAG with morphine,

glutamate, or electrical current inhibits spinal nociceptive neurons by activating intermediate nuclei in the rostral ventral medulla (RVM) that send projections to the spinal cord.

Supraspinal morphine stimulates three medullary nuclei

Studies using the autoradiographic method (Ruda, 1975) and the retrograde horseradish peroxidase transport technique (Gallager and Pert, 1978) have demonstrated the existence of projections from the ventrolateral PAG to the nucleus raphae magnus (NRM), the nucleus reticularis gigantocellularis (NRGC) and the nucleus reticularis paragigantocellularis (NRPG) of the medulla in the cat and the rat. Interconnections between these medullary nuclei were also noted. In addition, morphine injections to the PAG activate cells in the NRM (Behbehani, 1978 ; Fields, 1978). Evidence that these medullary nuclei are involved in opiate analgesia has come from many sources. Lesions in the NRM (Proudfit and Anderson, 1975; Proudfit, 1980; Yaksh et al.,1977; Samanin et al, 1970) and the NRGC (Mohrland, 1982) attenuated morphine-induced analgesia. Administration of morphine to the NRM, NRGC or the NRPG produced potent behavioral analgesia (Dickenson et al, 1979; Takagi et al., 1976 and 1977; Levy et al., 1979; Azami et al., 1982). There is also evidence for an enkephalinergic link between the PAG and these medullary nuclei. Naloxone administration to these nuclei attenuated morphine-induced analgesia (Jensen and Yaksh, 1986). Enkephalinergic cell bodies and terminals have been localized in the NRM, NRGC, and NRPG as well as the in the PAG (Hokfelt, 1977). Thus, supraspinal morphine probably induces analgesia by activating bulbospinal pathways mediated by these medullary nuclei.

Efferents from these medullary nuclei enter the spinal cord through the DLF

Basbaum et al. (1977) traced injections of tritiated leucine into the NRM, the NRGC and the NRPG . They found that the axon terminals of cells in these areas are located in the dorsal horn of the spinal cord in the rat. Another study using cats (Kuypers, et al, 1977) confirmed this finding. These studies provide neuroanatomical evidence for the spinopetal pathway described earlier. It is likely that supraspinal morphine activates cells in the ventrolateral PAG area that form synapses with cells in the NRM, NRGC and NRPG. Efferents from these medullary nuclei descend to enter the spinal cord via the DLF. They form synapses with primary pain afferents, (Aδ and C fibers) in the dorsal horn and inhibit their responses to noxious stimuli.

These medullary nuclei release monoamines that inhibit dorsal horn nociceptive neurons

Cells descending from the NRM to the spinal cord are predominantly serotonergic, while those descending from the reticular medullary nuclei are predominantly noradrenergic (Dahlstrom and Fuxe, 1965). The analgesic effects of morphine are probably modulated by the release of monoamines in the spinal cord that have an inhibitory effect on Aδ and C fibers in the dorsal horn. Several lines of evidence support the premise that spinal serotonin (5-HT) and norepinephrine (NE) modulate the effects of morphine-induced analgesia. Analgesia induced by morphine injections into the NRM were antagonized by intrathecal injections of the 5-HT antagonist methysergide but not by the α–NE antagonist phentolamine (Jensen and Yaksh, 1986). Conversely, microinjections of morphine into the NRGC and NRPG produced analgesia that was reversible by phentolamine but not by methysergide(Jensen and Yaksh, 1986). Iontophoretic administration of 5-HT and NE to the substantia gelatinosa of the spinal cord inhibited the discharge of nociceptive neurons that was reversed by monoamine antagonists but not by naloxone (Belcher et al., 1978, Headley et al., 1978). Intrathecal administration of 5-HT or α–NE antagonists, in the absence of morphine, elevated nociceptive thresholds in several species ranging from rat to primate (Reddy and Yaksh, 1980; Reddy et al., 1980; Yaksh and Wilson, 1979). Furthermore, morphine administration has been associated with increased turnover of serotonin (Shiomi et al., 1978) and norepinephrine (Shiomi and Takagi, 1974) in the spinal cord. Injections of morphine into the PAG caused the release of 5-HT from the spinal cord (Yaksh and Tyce,1979), whereas morphine injections into the NRGC induced NE release from the spinal cord. Collectively these studies provide strong evidence that supraspinal morphine may induce analgesia by activating spinopetal monoamine systems which serve to inhibit nociceptive neurons in the dorsal horn. Fields and Basbaum (1984) suggested that spinal monoaminergic neurons may synapse on enkephalinergic interneurons in the dorsal horn. According to their model, the release of 5-HT and NE by these spinopetal neurons may stimulate spinal interneurons to release enkephalins. These enkephalins may activate opiate receptors in the dorsal horn and induce analgesia by acting on the same neural substrates as are involved in analgesia induced by intrathecal opiate administration.

Differences in morphine-induced analgesia against thermal and mechanical noxious stimuli

Descending NE and 5-HT pathways may selectively modulate morphine-induced analgesia in response to different types of noxious stimuli. Depletion of spinal NE by intrathecal injections of the neurotoxin 6-OHDA attenuated the analgesic effects of morphine against mechanical but not thermal stimuli (Kuraishi et al, 1983). In contrast to this, depletion of

spinal NE using 5,6-DHT caused an attenuation of morphine-induced analgesia in response to thermal but not mechanical noxious stimuli. These authors also found that intrathecal administration of serotonin produced more robust analgesia against thermal noxious stimuli, while intrathecal administration of NE produced more profound analgesia against mechanical stimuli. Several studies showed that spinal NE modulated morphine analgesia against noxious thermal stimuli (Jensen, 1986; Sagen and Proudfit, 1984; Yaksh, 1979; Proudfit and Hammond, 1981). It may be that spinal NE modulates the analgesic effects of morphine against both mechanical and thermal nociceptive stimuli, although a more potent antinociceptive effect is seen in response to mechanical stimuli. The more pronounced modulation of mechanical analgesia by spinal NE might be most evident when the two types of stimuli are simultaneously compared.

Spinopetal NE and 5-HT systems may have a different developmental time course

Experiments performed in our laboratory demonstrated that morphine-induced analgesia against a thermal stimulus is measurable earlier in development than is analgesia against a mechanical stimulus (Giordano and Barr, 1987). This difference in ontogeny supports the premise that morphine-induced analgesia against thermal and mechanical stimuli may be modulated by distinct spinopetal monoamine systems. These spinopetal systems develop postnatally, and they do not develop at the same rate (Bregman, 1987; Simmons and Jones, 1985; Commissiong, 1983). Therefore it is not surprising that morphine-induced analgesia against thermal and mechanical stimuli do not develop at the same age. Morphine-induced analgesia was also found to have a rostral to caudal pattern of development. Analgesia was noted in the forepaw at an earlier age than in the hindpaw and tail (Giordano and Barr, 1987). These findings supports the premise that supraspinal morphine administration probably induces analgesia by activating spinopetal systems that also develop in a rostral to caudal manner: such as those descending to the spinal cord from the NRM, the NRPG and the NRGC.

Stimulation-Produced Analgesia

SPA and OA mediated by the PAG may share common mechanisms

The involvement of the PAG area in both SPA and OA suggests that both types of analgesia may be mediated by the same mechanisms, and there is some evidence for this. Electrical stimulation of the ventral PAG in the cat (Liebeskind et al, 1973) and the rat (Bennett and Mayer, 1976) inhibited the responding of nociceptive neurons in the dorsal

horn, indicating that SPA activates a bulbospinal pathway. Lesions of the NRM, the NRPG and the NRGC attenuated analgesia produced by PAG stimulation (Behbehani and Fields, 1979). Furthermore, SPA was reduced by depletion of 5-HT with parachloralphenyalanine (p-CPA). This reduction was reversed by the administration of the serotonin precursor 5-Hydroxytryptophan. Tetrabenzene, a compound that depletes all monoamines, almost totally abolished SPA, whereas increasing catecholamine levels with the catecholamine precursor L-DOPA potentiated SPA (Akil and Liebeskind, 1975).

The PAG can be stimulated chemically as well as electrically

The microinjection of small volumes of the excitatory neurotransmitter glutamate in the PAG also produced analgesia, increased neuronal activity in the PAG and increased the rate of firing of cells in the NRM (Behbehani and Fields, 1979; Urca et al., 1980; Jensen and Yaksh, 1984; Satoh et al., 1983). Thus, stimulation of the PAG either electrically or chemically will produce analgesia and will, to a certain extent, mimic the effects of intraPAG morphine.

Differences between the OA and SPA mediated by the PAG

Despite all of the apparent similarities between SPA, GPA and OA just mentioned, there are also many differences between these types of analgesia. Conflicting reports in the literature have provided doubt as to whether SPA and OA actually share a common mechanism of action. While SPA elicited from some PAG sites is reversed by the opiate antagonist naloxone (Oliveras et al, 1977), SPA elicited by other sites is not (Yaksh et al, 1976) or is incompletely (Akil et al, 1976) reversed by naloxone. The SPA supported by some PAG sites shows cross tolerance with morphine, while the SPA supported by other sites does not (Morgan and Liebeskind, 1987). Therefore, it appears that some sites within the PAG support a type of SPA that resembles OA, while other sites support SPA with a distinct mechanism of action.

Neuroanatomy of the PAG

The PAG is a complex structure that contains a diversity of cell types and communicates via afferent and efferent projections with a myriad of other central nervous system structures. The focus of this thesis is the role of the periaqueductal grey area in pain modulation. However, the function of the PAG in antinociception is inextricably linked to the neuroanatomical correlates that define this structure. Therefore, a brief overview of the neuroanatomy of the PAG will be presented in this section.

Cytoarchitectural subdivisions

This thesis examines differences between the dorsal and ventral PAG in the production of analgesia. Of interest, therefore, is whether the PAG has been cytoarchitecturally divided into a dorsal and ventral region. The cytoarchitectural subdivisions of the PAG will be reviewed in the following section.

Traditionally the PAG was divided into dorsal and ventral portions by the sulcus limitans (Castaldi, 1923). This division was based on the ontogenetic differentiation between the two parts rather than on cytoarchitectural differences. The dorsal portion of the PAG develops with the tectum whereas the ventral portion of the PAG develops with the tegmentum (Brown, 1943). Based on his study of guinea pigs, Castaldi (1923) divided the dorsal PAG into 3 parts, the single mediodorsal and the paired laterodorsal parts. The entire ventral area of the central grey was named the ventral nucleus and was subdivided into four parts: pars mediana dorsale and the pars mediana ventrale and the bilateral pars lateralis. Brown (1943) agreed that the pars dorsalis was an alar plate derivative and should be considered part of the central grey but did not differentiate nuclear subdivisions within it. He divided the subtectal portions into a pars lateralis which he described as cytoarchitecturally resembling the pars dorsalis and a pars ventralis.

Olszewski and Baxter (1954) divided the human PAG into three regions. They described a small dorsal subnucleus, a cellular outer mantle and an acellular inner mantle. Taber (1961) also described three areas in the grey based on topographical and cytological characteristics. These were dorsal, ventral and lateral subdivisions. The dorsal area contained numerous glial cells. The ventral area was characterized by sparsely distributed small cells. The lateral area was markedly cellular. Ramon-Moliner and Nauta (1966) described the PAG as part of the isodendritic core of the brain. The area was shown to be populated with

neurons with long axons that radiate in all directions. As is characteristic of isodendritic neurons, these were found to have undergone little evolutionary differentiation and to receive afferent input of multiple origin.

Hamilton (1973) characterized three distinct cell types within the cat PAG using cresyl violet nissl staining. Type I cells were small spindle shaped cells that stained darkly with cresyl violet. These cells were found to aggregate loosly around the cerebral aqueduct forming the comparative acellular inner ring of the PAG, the nucleus medialis. Type II cells were also small and darkly staining, but were fusiform to spherical in shape. These cells were clustered compactly and surrounded by many glial cells. They were found to congregate in the area dorsal to the aqueduct to form the nucleus dorsalis. Type III cells were the largest in the PAG. They were spherical or triangular in shape and contained little nissl substance. They were seen to aggregate closely to form the dense cellular outer portion of the PAG, the nucleus lateralis. Type III cells are thought to be the ones that contain the μ receptors to which morphine binds.

Lui and Hamilton (1980) subsequently divided the cells within the PAG into seven types based on the findings of a Golgi study. Cell types Ia and Ib were bipolar neurons with axons that projected beyond the PAG. Cell type II was a triangular cell with axons that also projected beyond the PAG. Cell types IIIa-d were pleiomorphic multipolar neurons. Cell types IIIa and IIId had axons that projected beyond the PAG. These are thought to be the neurons that project from the PAG to nuclei in the RVM and are important for the production of analgesia mediated by the PAG. The axons of cell types IIIb and IIIc terminated within the PAG. Type Ia cells were only found in the area immediately surrounding the aqueduct, the nucleus medialis. Type IIIc and IIId cells were found exclusively in the lateral regions of the PAG that correspond to the nucleus lateralis. The remaining cell types were distributed predominantly in the nucleus lateralis and the nucleus dorsalis.

Mantyh (1982) compared the cytoarchitecture of the PAG matter in the rat, cat, and monkey using both nissl staining and Golgi staining techniques. The nissl studies revealed a gradual increase in the intensity of cellular staining from the central portion of the PAG out toward the periphery of the PAG. This study demonstrated that the borders of the PAG were well defined except for the caudal ventral sites which were seen to merge with the dorsal raphae, a small nucleus that is embedded in the ventral aspect of the PAG at caudal levels. The Golgi studies showed that there are many cells and a dense plexus of small

diameter fibers in the central region of the PAG. These cells stained poorly with nissl stain and thus were not apparent in the nissl study. Furthermore, the Golgi studies demonstrated the presence of five major non-glial cell types in the PAG: A fusiform neuron that was most frequently found in the central region around the aqueduct, a multipolar neuron that was found in all PAG areas, a stellate cell that was evenly distributed throughout the PAG, a pyramidal cell that was seen in all areas of the PAG but was more numerous in the periphery than in the center of the PAG and ependymal cells that lined the aqueduct

The pyramidal cells noted by Mantyh were thought to correspond to the type III cells described by Hamilton. Therefore, it is these cells that are thought to mediate the analgesia supported by the PAG. The borders of the PAG clearly demarcated with the nissl stain were not well defined in Golgi stained neurons because of the spread of dendrites beyond the PAG and the presence of similar cell types in structures adjacent to the PAG. Mantyh (1982) also described the myeloarchitecture of the PAG. He found a gradual increase in fiber diameter, degree of myelination and cell size from the center to the periphery of the PAG. Moreover, the fiber systems change from a circular to a radial organization from the center to the periphery of the PAG. Mantyh (1982) found that the cytoarchitecture of the PAG was similar in the rat, cat, macaque and squirrel monkey and did not vary significantly when examined with nissl or Golgi methods. Therefore, he concluded that the PAG maintains a phylogenetic consistency in its basic structure and fiber connections.

Beitz (1985) used the cluster analysis technique to study the PAG. He described four major cellular subdivisions within the PAG. These were the dorsal , the dorsolateral, the ventrolateral and the medial subdivisions. These subdivisions were confirmed by the work of Conti et al. (1988) using cytochrome oxidase histochemistry. However, they found that these subdivisions were only present at caudal and middle levels of the PAG. At rostral levels only two concentric bands were seen, an inner band that showed low cytochrome oxidase activity and an outer, highly reactive band. These studies provide evidence for the neuroanatomical subdivision of the PAG into a dorsal and and a ventral region that may underlie functional differences betweeen these PAG regions.

There appears to be considerable variation in the cytoarchitecture of the PAG as described by various laboratories. Several consistent findings emerge as well. Several groups described changes in cell type and cell density from the center to the periphery of the PAG. In addition, there seem to be a number of different cell types within the PAG, some that are locally projecting neurons and others that project to structures external to the PAG. Cells

containing opiate receptors appear to be distributed throughout the PAG, but according to some accounts may be more concentrated in ventrapl or ventrolateral PAG regions. The cytoarchitecture of the PAG appears to remain relatively constant throughout the phylogenetic scale..

Peptidergic organization of the PAG

The role of the PAG in analgesia raises questions concerning the mechanism of pain modulation in this area. It is not clear, for example, if opiates, glutamate, or naloxone act on afferents to the PAG, on PAG projection neurons, or on PAG local ciruit neurons. The site of action of morphine within the PAG might be inferred, however, by the distribution of endogenous opiates or other peptides that are known to be involved in pain suppression. The following section will provide a brief review of what is known about the distribution of enkephalin, neurotensin and substance P peptides in the PAG.

Enkephalin

Early histochemical studies showed that enkephalinergic perikarya were only present in the caudal ventrolateral PAG areas in the rat (Hokfelt et al., 1977 a,b). Subsequent studies revealed a wider distribution but confirmed that enkephalinergic perikarya were more prevalent in ventrolateral PAG regions of the rat. Moss et al. (1983) found that the distribution of enkephalinergic perikarya and terminals in the cat was less uniform than in the rat. The concentration of enkephalinergic neurons in the cat underwent a ventral to dorsal shift from caudal to rostral sites within the PAG. They proposed that this ventral to dorsal shift in enkephalinergic immunoreactivity might either correspond to a somatotopic organization within the PAG or mirror the topographic organization of the PAG's interaction with other components of an endogenous analgesia system.

Neurotensin

The distribution of neurotensin-like immunoreactive neurons and fibers in the rat PAG was also examined using immunohistochemistry (Shipley, et al. 1987). Neurotensin immunoreactive fibers were found throughout the PAG, although they were more heavily distributed in the caudal half than in the rostral half of this region. They were also more numerous in the ventral half than the dorsal half of the PAG. Neurotensin immunoreactive fibers were heterogenously distributed, and were most concentrated in the caudal two thirds

of the PAG in the region adjacent to the aqueduct. These findings indicated that neurotensin acts predominantly on elements located in the medial and the ventrolateral PAG. The distribution of these fibers mirrored the sites at which exogenously applied neurotensin was shown to produce long lasting excitation of PAG neurons (Behbehani et al. 1987). Neurotensin activated neurons may project directly to the NRM and to other adjacent medullary nuclei (Behbehani and Pert, 1984; Williams and Beitz, 1989).

Substance P

The distribution of substance P ligand binding sites and immunoreactive neurons within the PAG has also been investigated (Lui and Swenberg, 1988). Binding sites were located using autoradiography and were shown to be more densely distributed in the dorsal half of the PAG than in the ventral half. Substance P containing neurons were located using immunohistochemistry. In the rostral PAG they were found throughout the entire dorsoventral region. In the caudal PAG they were located in three clusters: in the dorsolateral, dorsomedial and ventrolateral regions. Basbaum and Fields (1984) have suggested that substance P containing neurons within the PAG may act as interneurons within the local circuitry.

Enkephalinergic and neurotensin containing neurons were found to be predominantly localized in ventral regions of the PAG whereas neurons containing substance P are located mainly in dorsal PAG regions. The differential distribution of these peptides within the PAG probably is probably related in some way to the differences in analgesia supported by dorsal and ventral PAG regions. However, the precise nature of this relationship is not yet understood.

Projections to and from the PAG

The analgesia mediated by the PAG is thought to be subserved, at least in part, by a descending bulbospinal system. Ascending pathways may also contribute to the analgesia supported by the PAG. It is not known if cells from dorsal and ventral PAG sites project to the same nuclei within the RVM. If cells in the dorsal PAG project to or receive projections from different brain regions, this might account for differences in the analgesia supported by these regions. This section reviews the results of experiments that have traced ascending and descending projections to and from the dorsal and ventral regions of the PAG.

Efferent projections from the PAG

Hamilton & Skultety (1970) made electrolytic lesions in either the dorsal or ventral PAG. Degeneration in response to dorsal PAG lesions was in a radial pattern and extended to the superior colliculus, inferior colliculus and mesencephalic reticular formation. This degeneration was traced caudally to the cuneiform nucleus and adjacent reticular area. Rostrally, fibers travelled in the dorsolateral funiculus to the pretectal area, the lateral habenula nucleus and the posterior hypothalamic area. Ventral lesions showed the same radial pattern of degeneration. Caudally, the fibers could be traced to the cuneiform nucleus, the reticular area and the inferior olive. Rostrally, the course of fibers of the DLF was similar, though there were additional connections with the ventral tegmental area of Tsai, the fields of Forel and the parafascicular nucleus of the thalamus.

In 1974, Hamilton investigated the projection systems from the PAG in order to investigate differences in projections arising from three subdivisions of the PAG: the nucleus dorsalis, the nucleus medialis and the nucleus lateralis. She stereotaxically placed lesions in each of the three nucli and observed the resulting degeneration. Lesions of the nucleus dorsalis caused degeneration of axons projecting ventrally to the tegmental area, rostrally to the fields of Forel and the ventral tegmentum. Descending fibers were traced to the dorsal tegmental nucleus. Lesions of the nucleus dorsalis caused degeneration of a bundle of fibers that terminated at the mesencephaic-diencephalic junction in the pretectal area and the lateral habenular nucleus. Lesions of the nucleus lateralis caused degeneration of nuclei that terminated in the periventricular grey, the posterior hypothalamus and numerous thalamic nuclei.

Ruda (1976) studied the efferent projections of the PAG of the cat using ^{3}H leucine autoradiography. No difference in termination sites was noted between various anterior-posterior levels. Also, ascending projections were generally more dense than descending projections and ascending and descending fibers were seen to travel within the PAG. She also observed a discrete bundle of fibers that descended to the medullary levels. These fibers travelled adjacent to the pyramidal tract, crossed the trapezoid body and terminated within the medullary reticular formation. No direct projection to the spinal cord was observed. She also found significant and consistent changes between the projection of the dorsal and ventral aspects of the PAG. The major sites of termination of the dorsal PAG

were the nuclei parafascicularis, reuniens, posterior and paraventricular hypothalamus and the pontine and medullary reticular formation. The termination sites of cells in the ventral PAG were more extensive and included the midline nuclei, intralaminar nuclei, lateralis posterior, lateralis dorsalis, centralis centralis, fields of Forel, zona incerta, anterior, dorsal, posterior and lateral hypothalamic areas and the mesencephalic, pontine and medullary reticular formation. Ruda concluded, on the basis of these data, that the PAG could be divided into dorsal and ventral subnuclear groups that had different efferent projections.

In 1978, Gallager and Pert used microiontophoretically applied horseradish peroxidase to study the afferent projections to the raphae nuclei and reticular nuclei of the brainstem. They noticed that the afferents to the NRM originate mainly from structures rostral to the pons, such as the PAG and the dorsal and ventral tegmentum. In contrast, the NRGC received afferents primarily from more caudal structures such as the spinal cord grey matter. They also found interconnections between the raphae nuclei and reticular nuclei. They proposed that the NRM may serve as the connections with the medullary brainstem for a descending system, while the NRGC may be a relay in a feedback loop between the spinal cord and the reticular formation.

Watkins et al. (1981) used HRP slow release gels implanted into the dorsolateral funiculus of the spinal cord to study the nuclei that send axons through the DLF in rats. They found that cells within the rostral half of the PAG project through the DLF to the spinal cord, but that cells from the caudal half of the PAG do not send axons through the DLF. Beitz et al. (1983) localized bilateral projections from the ventral PAG to the spinal trigeminal nucleus.

Mantyh (1983) studied the ascending efferent projections of the PAG in the squirrel monkey using ^{3}H leucine autoradiograpy. He found that the PAG projects to the following diencephalic nuclei: the nucleus reticularis thalami, the nucleus medialis dorsalis, the midline thalamic nuclei, the intralaminar thalamic nuclei, the preoptic area, and the anterior, dorsal, periventricular, ventromedial, periarcuate, lateral and posterior hypothalamic nuclei. The main route for PAG-diencephalic projections was through the periventricular bundle. The zona incerta and the mesencephalic reticular formation also received projections from the PAG.

A short intrinsic circuit within the PAG has been noted using the Golgi technique (Tredici et al., 1983). Cells were noted with axonal collaterals that terminated on the somas of

neighboring neurons forming intrinsic circuits within the PAG. The axosomatic boutons were observed under the electron microscope and were found to be 1-1.5 μm in size, to contain pleiomorphic vesicles and to have symmetrical junctions. These morphological conditions are typical of inhibitory synapses. Therefore, these intrisic circuits are thought to be inhibitory and are likely to play a role in the tonic inhibition of cells within the PAG.

These tract tracing experiments indicate that while the efferents of the dorsal and ventral PAG overlap to a large degree, the projections of cells in the ventral PAG are generally more extensive than those arising from the dorsal PAG. Cells originating in the ventral but not the dorsal PAG project to the ventral tegmental area of Tsai, the fields of Forel, numerous thalamic and hypothalamic nuclei and to the spinal trigeminal nucleus. The more extensive projections of the ventral PAG could contribute to the ability of this region to support both SPA and OA in adult animals.

Afferent Projections to the PAG

Early studies utilized silver degeneration techniques to study projections to the PAG. The PAG has receives inputs from several rostral brain areas including numerous cortical sites (Goldman and Nauta, 1976; Hardy and Leichnetz, 1981), the hippocampus (Nauta,1958), the amygdala (Hopkins and Holstege, 1978), the hypothalamus (Conrad and Pfaff, 1976), the zona incerta (Ricardo, 1981), the superior colliculus (Graham, 1977) and the nucleus cuneiformis (Edwards, 1975). Nauta (1958) initially classified the PAG as part of the limbic midbrain area based on the extensive forebrain input to this region.

Mantyh (1982) used horseradish peroxidase to study forebrain projections to the PAG in the rat, cat and monkey. He observed projections to the PAG from the frontal cortex, the amygdala, the preoptic area and various hypothalamic nuclei. The main route for hypothalamic projections to the PAG was the periaqueductal bundle that immediately borders on the cerebral aqueduct. Projections to the PAG from the zona incerta, the mesencephalic reticular formation, the superior colliculus and the nucleus cuneiformis were also noted. The projections to the PAG appeared to be consistent in the three species examined. There did not seem to be differences in the projections to any of the subdivisions of the PAG previously decribed.

Meller and Dennis (1986) examined the projections to the PAG of the rabbit using horseradish peroxidase (HRP). The areas that were shown to project to the PAG in this study very closely matched those described by Mantyh (1982). However, labelling was found in some areas in this study that were not mentioned in the previous study. These areas included the substantia nigra, the parabigminal nucleus, the nucleus raphae magnus, medullary reticular nuclei and various thalamic nuclei. Furthermore, this study noted distinct differences in the projections to the dorsal, ventral and lateral aspects of the PAG. Areas that labelled most following ventral PAG HRP administration were the medial and lateral preoptic nuclei, the substantia nigra and the cells in the preolivary zone and the caudal raphae nuclei. HRP injections into the lateral PAG caused labelling predominantly in the dorsal, anterior and ventromedial hypothalamus, whereas dorsal PAG injections most effectively labelled cells in the ventromedial and anterior hypothalamus, the nucleus suprageniculatus and the parabigeminal nucleus. Differences in labelling were also noted when HRP was given to rostral or caudal PAG sites. Similar topographic differences in the projections to the PAG were noted by Beitz in the rat (1982). Differential projections to dorsal and ventral PAG sites might also contribute to differences in the ability of these regions to support different types of analgesia.

Goals of this thesis

The goal of this thesis was to clarify the similarities and differences between OA and GPA in the dorsal and ventral PAG by utilizing the developmental paradigm. Presumably, if GPA elicited by the dorsal and ventral PAG are mediated by distinct neuroanatomical substrates, they might not develop uniformly. In addition, if they are not mediated by the same spinal monoamine systems, the effects of spinally administered 5-HT and NE agonists and antagonists on these systems would not be the same during development. Furthermore, if GPA and OA in the ventral PAG were mediated by distinct neurons or receptor types, they may not have the same developmental pattern, and could be distinguished developmentally.

This study consisted of three groups of experiments. The first set compared the analgesic effects of glutamate administered to the dorsal and ventral PAG regions with those of morphine administered to the dorsal and ventral PAG in 3, 10, and 14 day old rat pups against noxious thermal and mechanical stimuli. The second set of experiments studied the effects of systemic administration of the opiate antagonist naloxone on morphine-induced and glutamate-produced analgesia mediated by the dorsal and ventral PAG of 10 and 14

day old rat pups. The third set of studies combined the same glutamate and morphine intraPAG microinjections with the intrathecal administration of the noradrenergic antagonist phentolamine and the serotonergic antagonist methysergide in 14 day old rat pups in order to determine the involvement of spinal NE and 5-HT on OA and GPA.

Experiment 1

Microinjections of morphine into the PAG, extending from the region surrounding the rostral portions of the fourth ventricle, through the area surrounding the cerebral aqueduct, into the regions around the caudal aspect of the third ventricle, induced behavioral analgesia as measured by many different types of standard tests including the tail flick test, the hot-plate test, the flinch jump test and the pinch test (Criswell, 1976; Herz, 1970; Tsou and Jang, 1964; Jaquet, 1974, Lewis and Gebhart, 1977; Mayer and Murfin, 1976; Sharpe et al., 1974). Morphine aministered to the PAG induced measurable behavioral analgesia regardless of which assay was used.

Electrical stimulation of the PAG also produces analgesia. Electrical stimulation of medial brainstem sites produced behavioral analgesia in rats (Balagura and Ralph, 1973; McCreery and Bloedel, 1975; Mayer et al, 1971; Soper, 1976; Cannon et al., 1982; Fardin et al., 1984; Nichols et al., 1989) cats (Liebeskind et al., 1973; Melzack and Melinkoff, 1974; Oliveras et al., 1974) rhesus monkey (Goodman and Holcombe, 1976; Ruda et al., 1976) and humans (Gybels et al., 1976; Hosobuchi et al., 1977; Richardson and Akil, 1977).

The excitatory amino acid glutamate also produced analgesia when microinjected into the PAG (Urca et al., 1980; Jensen and Yaksh, 1984,1989). Unlike electrical stimulation, which excites both cell bodies and axons of passage within the PAG, the excitatory effect of glutamate is limited to cell bodies (Goodchild et al., 1982). Glutamate receptors were identified within the PAG using autoradiography (Greenamyre et al., 1983). When the glutamate receptor agonist NMDA was administered to the PAG, it produced potent analgesia against noxious thermal and mechanical stimuli (Jaquet, 1988). Kainic acid and quisqualic acid were shown to produce explosive motor behaviors as well as analgesia when administered to the PAG. These findings indicated that specific stimulation of any one of the the three types of glutamate receptors within the PAG has behavioral effects. Therefore, it appears that the three types of opiate receptors are present and functional within the PAG (Jaquet and Squires, 1988).

Attempts to reverse the analgesia produced by intraPAG glutamate have yielded ambiguous results. The analgesic effects of glutamate administration to the PAG were either reversed or potentiated by systemic naloxone (Urca et al.,1980) and unaffected by intrathecal naloxone administration (Jensen and Yaksh, 1984). The analgesia produced by NMDA administration to the PAG was not reversed by the opiate antagonist naloxone indicating that the analgesia produced by NMDA is independent of opioid activity (Jaquet, 1988).

The analgesic effects of morphine and glutamate administration to coincident sites within the periaqueductal grey were recently compared in adult rats. The analgesic effects of both compounds were indistinguishable (Jensen and Yaksh, 1989). A comparison of the analgesic effects of morphine and glutamate administration to nuclei in the rostral ventral medulla (RVM), showed that the analgesia induced by morphine, but not by glutamate, displayed a plateau (Jensen and Yaksh, 1989). These findings suggested that medullary nuclei may differentially mediate the analgesia produced by these compounds. The first goal of this study was to determine if there are developmental differences in the morphine-induced or glutamate-produced analgesia supported by the PAG.

Stimulation of the PAG with morphine, glutamate or electrical current is thought to induce analgesia by causing the release of monoamine neurotransmitters in the spinal cord and thereby inhibiting incoming noxious impulses. Direct projections from the PAG to the spinal cord do exist (Kuypers and Maisky, 1975, 1977; Kniesley, 1978; Leichnetz et al.,1978). Because these projections are relatively sparse (Ruda, 1975; Kuypers, 1975) it is thought that stimulation of the PAG effects spinal nociceptive neurons by activating intermediate nuclei in the rostral ventral medulla that send projections to the spinal cord. These medullary nuclei are the NRM, the NRGC and the NRPG. These nuclei all receive projections from the PAG (Ruda, 1975; Gallager and Pert, 1978; Pomeroy and Behbehani, 1979; Abols and Basbaum,1981) and send projections through the DLF to layers of the spinal cord dorsal horn that contain second order pain transmission cells and the terminals of nociceptive primary afferents (Basbaum et al. 1978; Cervero et al. 1977; Kuypers, 1975; Dahlstrom and Fuxe, 1965).

The first evidence of a bulbospinal system descending from the PAG to nuclei in the RVM and projecting from these nuclei to the spinal cord dorsal horn came from lesion studies. The antinociceptive effects of systemically administered morphine on the tail flick reflex were attenuated in animals with transsected spinal cords (Irwin et al., 1951) whereas the

analgesia produced by electrical stimulation of the PAG was reversed by subtotal spinal cord lesions (Basbaum et al., 1977). OA and SPA supported by the PAG were abolished by lesions or blockade of the DLF (Sandkuhler et al., 1987; Murfin et al., 1976). The antinociceptive effects of systemically administered morphine were partially antagonized by DLF lesions (Basbaum et al., 1977; Hayes et al., 1978; Price et al., 1976). Moreover, lesions of the NRM, the NRPG or the NRGC have been shown to attenuate the analgesia produced by systemically administered morphine, intraPAG morphine or PAG stimulation (Behbehani and Fields, 1979; Sandkuler and Gebhart, 1984; Proudfit and Anderson, 1980; Yaksh et al., 1977; Samanin et al., 1970; Mohrland et al., 1982). Lesions of the NRM abolished electrically induced SPA from ventral but not dorsal sites within the PAG (Prieto, et al., 1983). However, when cells within the dorsal PAG were stimulated using glutamate the resulting analgesia was disrupted by NRM lesions (Behbehani and Fields, 1983). This indicates that the mechanisms of action of chemical and electrical stimulation of the PAG in the production of analgesia are not the same although they may overlap to some degree. Collectively these experiments support the functional integrity of a pathway originating in the PAG and descending to three monoaminergic nuclei in the RVM and from these nuclei to the DLF of the spinal cord in the modulation of morphine-induced analgesia.

As a rule, cells descending from the NRM to the spinal cord are serotonergic, whereas those descending from the reticular medullary nuclei are noradrenergic (Dahlstrom and Fuxe, 1965). However, serotonergic perikarya have been localized in the NRPG and noradrenergic and dopaminergic perikarya have been found within the NRM (Beitz, 1982; Bowker, 1982). The analgesia evoked by administering morphine to the NRM, or stimulating this nucleus electrically or with glutamate was reversed by intrathecal administration of methysergide (Satoh et al., 1983; Hammond and Yaksh, 1984; Jensen and Yaksh, 1986) whereas morphine-induced and electrical and glutamate stimulation-produced analgesia supported by the NRPG and the NRGC were reversed by intrathecal administration of phentolamine (Satoh et al., 1980; Jensen and Yaksh, 1986b). Electrical stimulation of the NRM has been shown evoke the release of serotonin into spinal cord superfusates (Hammond et al., 1985) while electrical stimulation of the NRPG has been shown to induce the release of NE into spinal cord superfusates (Hammond et al., 1985). Thus, stimulation of the NRM seems to induce the release of serotonin into the spinal cord while stimulation of the reticular nuclei induce the release of norepinephrine into the spinal cord. Stimulation of the PAG electrically or using morphine or glutamate is thought to activate cells that project to the NRM, the NRPG and the NRGC. These cells, in turn,

causes the release of both monoamine neurotransmitters into the spinal cord and a resultant inhibition of Aδ and C fibers in the dorsal horn.

Electrophysiological and pharmacological evidence supports the premise that spinal 5-HT and NE modulate the effects of morphine-induced and stimulation-produced analgesia mediated by the PAG. Stimulation of the PAG electrically or with glutamate or morphine inhibits the responding of nociceptive Aδ and C fibers in the dorsal horn of the spinal cord (Bennett and Mayer, 1976; Liebeskind et al., 1973; Sandkuhler, 1988). Morphine-induced, glutaminergic, and stimulation-produced analgesia mediated by the PAG have all been reversed by intrathecal administration of the α-noradrenergic antagonist phentolamine and the serotonergic antagonist methysergide but not by the opiate antagonist naloxone (Jensen and Yaksh, 1984, 1986b; Aimone et al., 1987; Satoh et al., 1983). SPA supported by the PAG has been reduced by depletion of 5-HT with parachloralphenyalanine (p-CPA) and this reduction was reversed by the administration of the serotonin precursor 5-Hydroxytryptophan. Tetrabenzene, a compound that depletes all monoamines, almost totally abolished SPA, whereas increasing catecholamine levels with the catecholamine precursor L-DOPA potentiated SPA (Akil and Liebeskind, 1975). Morphine administration to the PAG was associated with increased turnover of 5-HT (Shiomi et al., 1978) and NE (Shiomi and Takagi, 1974) in the spinal cord. IntraPAG morphine caused the release of 5-HT into the spinal cord (Yaksh and Tyce, 1979). In addition, electrical stimulation of the PAG caused the release of 5-HT and NE into spinal cord superfusates in the rat (Hammond et al., 1985). Furthermore, morphine microinjections into the PAG increased the rate of 5-HT synthesis in the spinal cord (Yaksh and Tyce, 1979). Iontophoretic administration of 5-HT and NE to the substantia gelatinosa of the spinal cord inhibited the discharge of nociceptive neurons in the dorsal horn. This inhibition was reversed by monoamine antagonists but not by naloxone (Belcher et al., 1978; Headley et al., 1978). Moreover, intrathecal administration of 5-HT or NE antagonists, in the absence of supraspinal stimulation or morphine administration, elevated nociceptive thresholds in several species at different ages (Reddy and Yaksh, 1980; Reddy et al. 1980; Yaksh and Wilson,1979; Hughes and Barr,1988).

The PAG has been functionally subdivided into a dorsal and a ventral region. The ventral region is thought to support an morphine-induced analgesia as well as SPA, whereas the dorsal PAG is thought to support SPA but not morphine-induced analgesia. This division was originally noted when SPA arising in the PAG was reversed (Oliveras et al., 1977), partially reversed (Akil et al., 1976), or not reversed at all (Yaksh et al., 1976) by the

opiate antagonist naloxone. Naloxone elevated thresholds for PAG stimulation needed to produced analgesia from ventral but not from dorsal sites. Cannon et al. (1982) and Fardin et al. (1984) both observed that the analgesia following the termination of PAG stimulation (post stimulation analgesia) persisted longer in dorsal regions of the PAG. In addition, a continuous spectrum of nociceptive thresholds was observed when the ventral PAG was stimulated, while responses in the dorsal PAG were quantal. Cross tolerance was demonstrated between electrical SPA and morphine-induced analgesia supported by ventral but not dorsal sites in the PAG (Morgan and Liebeskind, 1987). The functional differences found between the dorsal and the ventral PAG may be a reflection of anatomical differences between these areas. In vitro receptor binding studies have demonstrated a high density of opiate binding sites within the ventral PAG and fewer within dorsal aspects of the PAG (Beitz et al.,1982). In contrast to this, glutamate receptors are more numerous within the dorsal PAG than the ventral PAG (Beitz et al., 1985). Dynorphin-like peptides are found in higher concentrations in the ventral PAG (Beitz et al., 1985). Furthermore, the dorsal and ventral PAG receive differential projections from other areas of the brain. Lateral hypothalamic and cortical projections to the PAG terminate predominantly in ventral PAG regions, whereas projections from the zona incerta and the ventromedial hypothalamus terminate primarily in dorsal PAG areas. Since it is known that three receptor types for glutamate are present in the PAG it is possible that differential distribution of these receptor populations combined with the differential ontogeny of these receptor types (Slevin and Coyle, 1981) might allow glutamate to produce analgesia when given to some sites within the PAG than to others. The differences in the analgesia supported by the dorsal and ventral aspects of the PAG may have their basis in some of these anatomical distinctions. The second goal of this study was to determine whether glutamate-produced analgesia and morphine-induced analgesia are differentially mediated by the dorsal and ventral PAG in developing animals.

There is some evidence that noradrenergic spinal afferents modulate analgesia against mechanical noxious stimuli to a greater degree than thermal noxious stimuli. The converse may be true for serotonergic spinal afferents. The work of Kuraishi et al. (1983) provided the first direct evidence for this. Depletion of spinal NE by intrathecal injections of the neurotoxin 6-hydroxydopamine (6-OHDA) attenuated the analgesic effects of morphine against mechanical but not thermal stimuli. In contrast to this, depletion of spinal 5-HT using 5,6-dihydroxytryptamine (5,6-DHT) caused an attenuation of morphine-induced analgesia in response to thermal, but not mechanical noxious stimuli. Intrathecal administration of serotonin produced more robust analgesia against thermal noxious

stimuli, and intrathecal administration of NE produced more profound analgesia against mechanical stimuli in adult rats (Kuraishi et al.,1985). Experiments performed in developing rats supported these findings. Intraspinal 5,7-dihydroxytryptamine attenuated the analgesic effects of systemic morphine against a thermal stimulus to a greater degree than analgesia against a mechanical stimulus (Giordano and Barr, 1988). Intrathecal administration of norepinephrine or the α_2 noradrenergic agonist clonidine resulted in more pronounced analgesia against a mechanical than a thermal stimulus (Hughes and Barr, 1988). It is possible that cells within the dorsal and ventral aspects of the periaqueductal grey may differentially project to serotonergic or noradrenergic nuclei within the rostral ventral medulla. Thus, stimulation of some sites witin the PAG with glutamate or morphine may produce more pronounced analgesia against one type of noxious stimulus than another. The third goal of this experiment was to compare the analgesic effects of glutamate or morphine given to the dorsal or the ventral PAG of developing animals against thermal and mechanical noxious stimuli.

Systemically administered morphine produces dose dependent analgesia that develops in a rostral to caudal direction along the body of the animal (Giordano and Barr, 1987). This rostral to caudal development may mirror the growth of descending monoaminergic bulbospinal pathways that originate in the RVM. These spinal monoamine systems develop postnatally and they do not develop at the same rate (Bregman, 1987; Simmons and Jones et al.,1985; Commissiong, 1983). Thus, morphine given systemically may produce analgesia by acting on receptor sites within the PAG and thereby activating the three medullary nuclei from which these spinopetal monoaminergic fiber systems originate. If this is the case, it is likely that the development of analgesia produced by morphine administration to or stimulation of the PAG might parallel, to some extent, the development of analgesia induced by systemic administration of morphine. The fourth goal of this experiment was to determine whether morphine-induced or glutamate-produced analgesia supported by the PAG develops in a rostral to caudal direction.

The first experiment in this study compared analgesia produced by glutamate and morphine administration to the dorsal and ventral aspects of the PAG in developing rats. Differences in the ontogeny of morphine-induced and glutamate-produced analgesia could indicate that these compounds have different modes of action within the PAG or that they differentially activate efferent systems originating there. There were four major hypotheses tested in this experiment. The first was that glutamate-produced analgesia and morphine-induced analgesia supported by the PAG might have a differential ontogeny despite their similarity

in the adult animal. The second was that the PAG can be functionally divided into a dorsal and ventral region with regard to GPA and OA in the developing animal. The third was that there are differences in the analgesic effects of intraPAG glutamate and morphine against thermal and mechanical noxious stimuli in the developing animal. The fourth was that the opiate and non-opiate analgesia supported by the PAG develops in a rostral to caudal direction as does the analgesia induced by systemic morphine.

Methods

Subjects

Subjects were the offspring of Long Evans hooded rats mated in our laboratory. Pregnant rats were checked for newborn pups twice daily. When discovered for the first time, pups were termed age 0. Dams and their litters were housed in 40 X 20 X 24 cm plastic cages in a temperature and humidity controlled room. The light dark cycle was maintained at 12:12. Food and water were constantly available. Litters were reduced to 12 pups on day 3, but were otherwise not interfered with until pups were removed for surgery. Litters were considered experimental units. Litters were permanently removed from the dam at either age 3, 10, or 14 days. These ages were chosen because there are distinct changes in the analgesic response to morphine at these ages and morphine-induced analgesia is mostly developed in the rat by day 14 (Giordano and Barr, 1987).

PAG cannula implants

Six littermates were removed from the dam at the appropriate age and placed in an incubator maintained at 33^{0} C until each pup was individually removed for surgery. Pups were weighed and marked with indelible ink for identification purposes and anesthetized by inhalation of methoxyflurane (Metafane, Pitman-Moore). The dorsal-ventral coordinates of the cannula implant were calculated using the dura as a reference. There were two distinct dorsal-ventral coordinates at each age, one for the dorsal PAG and one for the ventral PAG. The PAG implant coordinates that were used for each age are shown in Table 1.

Table 1. Dorsal and ventral PAG coordinates relative to bregma in pups aged 3, 10 and 14 day old rat pups.

Age in Days	A-P	M-L	Dorsal	Ventral
Three	-2.9	+0.3	-1.8	-2.1
Ten	-5.5	+0.5	-2.0	-2.5
Fourteen	-6.0	+0.7	-1.8	-2.0

These coordinates correspond to the dorsal and ventral aspects of the PAG at the level of the dorsal raphae nucleus. The stereotaxic coordinates for the 10 and 14 day old pups were determined using the Sherwood and Timeras atlas (1970) as a starting point. Coordinates based on the information available had to be "fine tuned" by trial and error, using three or four subjects for each age, and continually verifying the implant sites until the implant was at the desired site. A 26 ga. 460 μm guide cannula manufactured by Plastic Products was lowered to 1 mm above the final injection site. The implanted cannulae were anchored to the skull using a thin layer of caulk grip resin cement followed by a thicker layer of acrylic cement. Once the acrylic cement had fully hardened, a stylet was placed through the guide cannulae and screwed in place. Pups were returned to the incubator for a recovery period of approximately 20 hours.

PAG Injections

Pups were removed from the incubator and weighed. Baseline withdrawal responses were measured for each pup. A 33 ga. 200 μm injector cannula, manufactured by Plastic Products, was connected at one end to polyethylene tubing. The tubing was connected at the other end to a Hamilton 1 μl syringe. The stylet was removed from the guide cannula, and the injector cannula was inserted through the guide cannula and fastened in place. Over a sixty second period, 0.2 μl of drug was injected by hand. The doses of morphine given were vehicle, 2 μg or 6 μg salt weight. The concentrations of glutamate given were vehicle, 60 mM or 180 mM. The injector cannula was left in place for an additional 60 seconds and then removed. The stylet was then replaced within the guide cannula. Analgesic testing was performed 2 minutes after glutamate treatment and 10 minutes after morphine treatment as these were empirically determined to be the times of optimal responding for the ages tested in this experiment. Following testing, 0.2 μl of India ink was injected through the guide cannula using the procedure just described. This was done to assist in the verification of the implant site and to approximate the extent of spread of the drug within the PAG.

Analgesia Testing Procedures

Testing was done both prior to and following administration of drugs. The withdrawal latency of the forepaw, hindpaw and tail of each animal from noxious thermal and

mechanical stimuli was measured to 1/100 of a second using a timer manufactured by Lafayette instruments that was operated using a foot pedal.

For the mechanical test, a 64 gram weight with a 0.2 cm diameter flat surface was gently applied in turn to the dorsal surface of the forepaw, hindpaw, and tail. Contact was made between the 0.2 cm surface and each limb before allowing the weight to be released in order to assure that withdrawal responses were not made to tactile non-noxious stimuli. The timer was activated upon release of the weight onto the limb. A response was deemed a withdrawal response when the animal removed its limb from underneath the weight. At this moment the timer was stopped by release of the foot pedal. In some instances very young animals (eg. 3 day old pups) were not strong enough to fully retract their limbs from the weight. Under these circumstances withdrawal from the stimulus was determined to be the first visible attempt to remove the limb even if the attempt was not successful.

For the thermal test, the forepaw, hindpaw, and tail were individually submerged in a water bath maintained at 47^{o} C. Animals were held suspended above the bath and only the limb or tail being tested was submerged into the water. The timer was activated at the moment the limb or tail hit the surface of the water and turned off the moment the limb or tail was removed from the bath by the animal. In this manner, withdrawal latencies were measured as the latency to fully withdraw the limb or tail from the bath.

The order of presentation of stimuli to each limb and the order of mechanical and thermal stimulus presentation were randomly determined for each pup. Each pup was tested only one time before and one time following drug administration. A 10 second cutoff latency was used for both tests after which the stimulus was discontinued even if there had been no response.

Histology

Pups were perfused with 0.9% saline, immediately followed by perfusion with 10% formaldehyde. Animals were decapitated and brains removed and stored in 70% alcohol until they were dehydrated, embedded in paraffin, blocked, and sectioned in the area of the PAG. Ten μm sections were cut and placed on gelatin treated slides. Slides were stained with Cresyl Violet, coverslipped, and examined under a light microscope to verify PAG implant sites.

Experimental Design

In this experiment, 3, 10 and 14 day old pups were given intraPAG injections of one of three doses of either morphine or glutamate administered to either the dorsal or ventral aspects of the PAG and tested for analgesic responses to mechanical and thermal stimuli. Experimental units for each age consisted of six littermates that were assigned to either the morphine or the glutamate condition. Three pups in each unit were given ventral PAG cannula implants and three were given dorsal PAG implants. Experimental units of six littermates that were assigned to the morphine condition pups were treated in the following manner. One pup with a ventral PAG implant and one pup with a dorsal implant received an intraPAG injection of 2 μg of morphine (N=2). One pup with a ventral PAG implant and one pup with a dorsal PAG implant received an intraPAG injection of 6 μg of morphine (N=2). One pup with a ventral PAG implant and one pup with a dorsal implant served as controls and received saline vehicle (N=2). Experimental units of six littermates that were assigned to the glutamate condition pups were given the following drug treatments. One pup with a ventral PAG implant and one pup with a dorsal implant received an intraPAG injection of 60 mM of glutamate (N=2). One pup with a ventral PAG implant and one pup with a dorsal implant received an intraPAG injection of 180 mM of glutamate (N=2). One pup with a ventral PAG implant and one pup with a dorsal implant served as a control and received saline vehicle (N=2). Six experimental units were used for each age (Total number of subjects was therefore 6 per unit x 6 units x 3 ages =108). Pups in all conditions were tested for nociceptive responding using the methods described earlier.

Statistics

The data were analyzed using a six way analysis of variance. The between litter factors were drug (morphine or glutamate) and age (3, 10 or 14 days). Within litter variables were implant site (dorsal or ventral) and dose (control, moderate, or high). Within subject factors were body part (forepaw, hindpaw, tail). For the purpose of the analysis of variance, both the within litter variables and the within subject variables were considered within group variables. Six litters were used for each drug, at each age. The factorial interactions of interest in this experiment were dorsal vs.ventral drug effects and the morphine vs. glutamate analgesic effects at the various ages tested. A post hoc Tukey test was done to ascertain the significance of the interactions.

Results

Spread of Drug Within the PAG

Brains were examined under the light microscope to determine the spread of India ink within the PAG. The degree of ink spread throughout the PAG was thought approximate the spread of drug throughout this area. India ink staining was limited to discrete sites within the PAG that were just ventral to the termination of the cannula tract. No ink was seen in sites external to the PAG with the exception of some sites within the dorsal raphae nucleus (DRN). The DRN is embedded into the ventralmost portion of the PAG caudal levels. Implants that fell within the DRN were considered ventral PAG implants for the purposes of this study. Data obtained following drug administration DRN did sites did not differ significantly from data obtained following drug administration to ventral PAG sites. Therefore, grouping these two types of implant sites together for the purpose of our data analysis seemed appropriate. The complete results of the ANOVA for this expriment are shown in Table 2.

Table 2. This table show the results of the analysis of variance for Experiment 1. Probabilities are shown for all interactions. Abbreviations are as follows: A is age (3,10,14 days), C is compound (morphine or glutamate), P is area of the PAG (dorsal or ventral), S is stimulus (thermal or mechanical), D is dose of drug (vehicle, low or high) and L is location of stimulus on the body (forepaw, hindpaw or tail).

Source	Sum of Squares	Degrees of Freedom	Mean Square	F	Tail Probability
A	2651.71	2	1325.85	1417.29	0.0000
C	94.65	1	94.65	101.18	0.0000
AC	291.22	2	145.61	155.65	0.0000
P	221.08	1	221.08	69.30	0.0000
PA	152.48	2	76.24	23.90	0.0000
PC	341.74	1	341.74	107.12	0.0000
PAC	127.97	2	63.99	20.06	0.0000
S	7.12	1	7.12	12.54	0.0013
SA	27.28	2	13.64	24.02	0.0000
SC	80.26	1	80.26	141.38	0.0000
SAC	17.81	2	8.91	15.69	0.0000
PS	28.87	1	28.87	90.47	0.0000
PSA	31.47	2	15.74	49.31	0.0000
PSC	10.71	1	10.71	33.56	0.0000
PSAC	20.53	2	10.26	32.16	0.0000
D	1798.80	2	899.40	658.69	0.0000
DA	1621.44	4	405.36	296.87	0.0000
DC	62.76	2	31.38	22.98	0.0000
DAC	161.27	4	40.32	29.53	0.0000
PD	156.42	2	78.21	52.19	0.0000
PDA	133.90	4	33.47	22.34	0.0000
PDC	214.59	2	107.30	71.60	0.0000
PDAC	65.76	4	16.44	10.97	0.0000
SD	5.08	2	2.54	5.74	0.0052
SDA	15.19	4	3.80	8.59	0.0000
SDC	58.82	2	29.41	66.49	0.0000
SDAC	14.81	4	3.70	8.37	0.0000
PSD	20.16	2	10.08	18.04	0.0000
PSDA	28.93	4	7.23	12.94	0.0000
PSDC	6.08	2	3.04	5.44	0.0067
PSDAC	12.61	4	3.15	5.64	0.0006
L	18.22	2	9.11	17.12	0.0000
LA	8.56	4	2.14	4.02	0.0059
LC	2.99	2	1.49	2.81	0.0683
LAC	4.82	4	1.20	2.26	0.0727
PL	8.29	2	4.15	9.70	0.0002
PLA	4.28	4	1.07	2.50	0.0516
PLC	13.64	2	6.82	15.96	0.0000
PLAC	9.16	4	2.29	5.36	0.0009

SL	6.27	2	3.14	9.80	0.0002
SLA	3.47	4	0.87	2.71	0.0384
SLC	12.23	2	6.11	19.10	0.0000
SLAC	3.60	4	0.90	2.81	0.0332
PSL	15.85	2	7.92	25.46	0.0000
PSLA	1.79	4	0.45	1.44	0.2314
PSLC	5.73	2	2.86	9.20	0.0003
PSLAC	1.26	4	0.31	1.01	0.4077
DL	9.43	4	2.36	4.53	0.0019
DLA	7.64	8	0.95	1.83	0.0773
DLC	1.40	4	0.35	0.67	0.6120
DLAC	7.24	8	0.90	1.74	0.0966
PDL	3.50	4	0.87	2.53	0.0441
PDLA	6.10	8	0.76	2.21	0.0314
PDLC	8.55	4	2.14	6.18	0.0001
PDLAC	10.09	8	1.26	3.65	0.0008
SDL	2.55	4	0.64	1.72	0.1505
SDLA	4.27	8	0.54	1.44	0.1875
SDLC	5.91	4	1.48	3.98	0.0046
SDLAC	6.47	8	0.81	2.18	0.0336
PSDL	10.67	4	2.67	8.29	0.0000
PSDLA	4.83	8	0.60	1.88	0.0699
PSDLC	2.70	4	0.68	2.10	0.0850
PSDLAC	3.31	8	0.41	1.28	0.2580

Morphine Effects

General observations: Morphine injections were made into a total of 18 dorsal and 18 ventral sites within the PAG at each age. The sites of injection for each age can be seen in Figure 1. In 3 day old rat pups, morphine-induced significant levels of analgesia when administered to the ventral PAG. This analgesia was most potent against the thermal stimulus and was seen only in the forepaw. In 10 day old pups, the PAG site and the stimulus specificity of morphine-induced analgesia persisted, while the analgesic effects of morphine became evident in caudal body parts as well. In 14 day old pups, morphine-induced analgesia was noted in all body parts. Analgesia was induced when morphine was given to either the dorsal or the ventral PAG and was seen against both types of stimuli. Graphic representations of these data are shown in Figure 2.

Figure 1. PAG sites into which morphine was administered in 3, 10 and 14 day old rat pups.

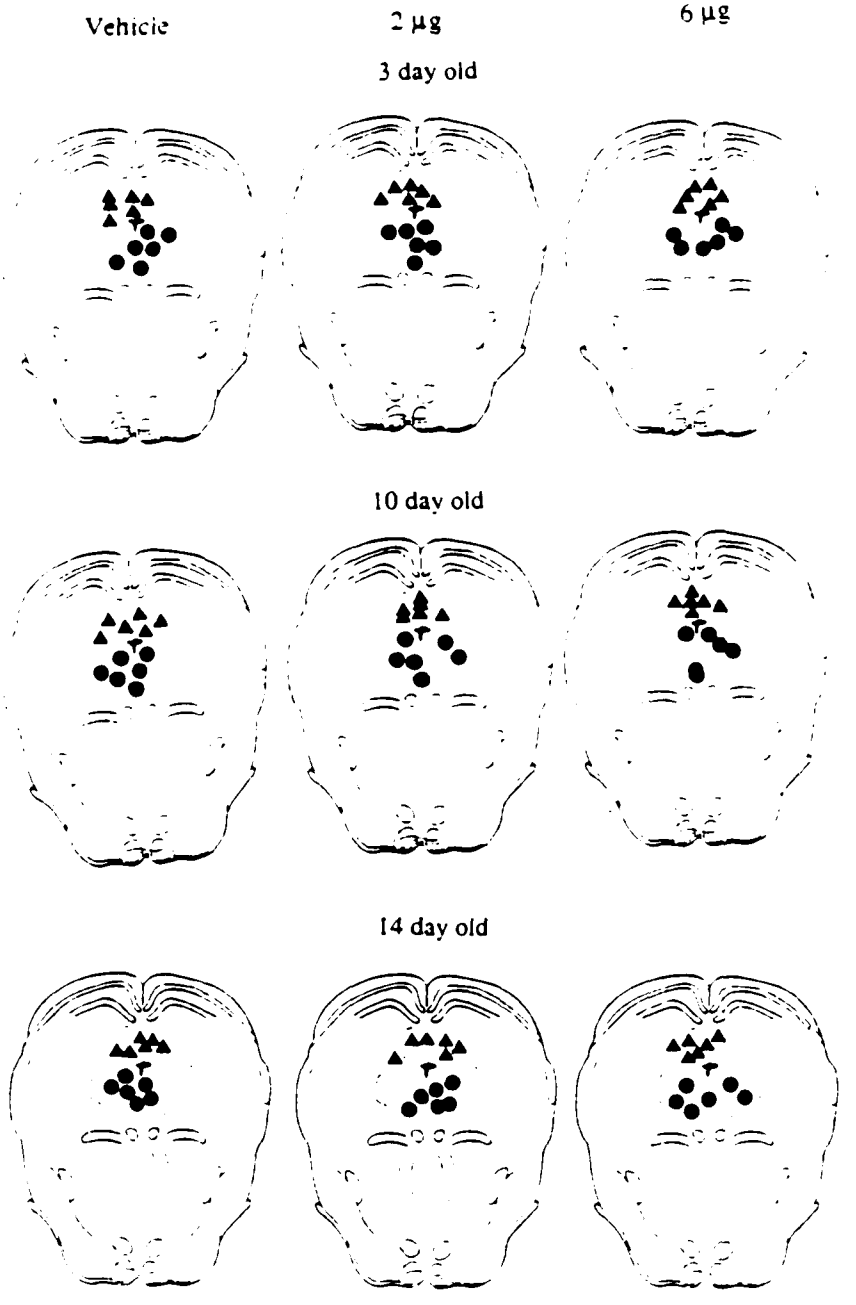
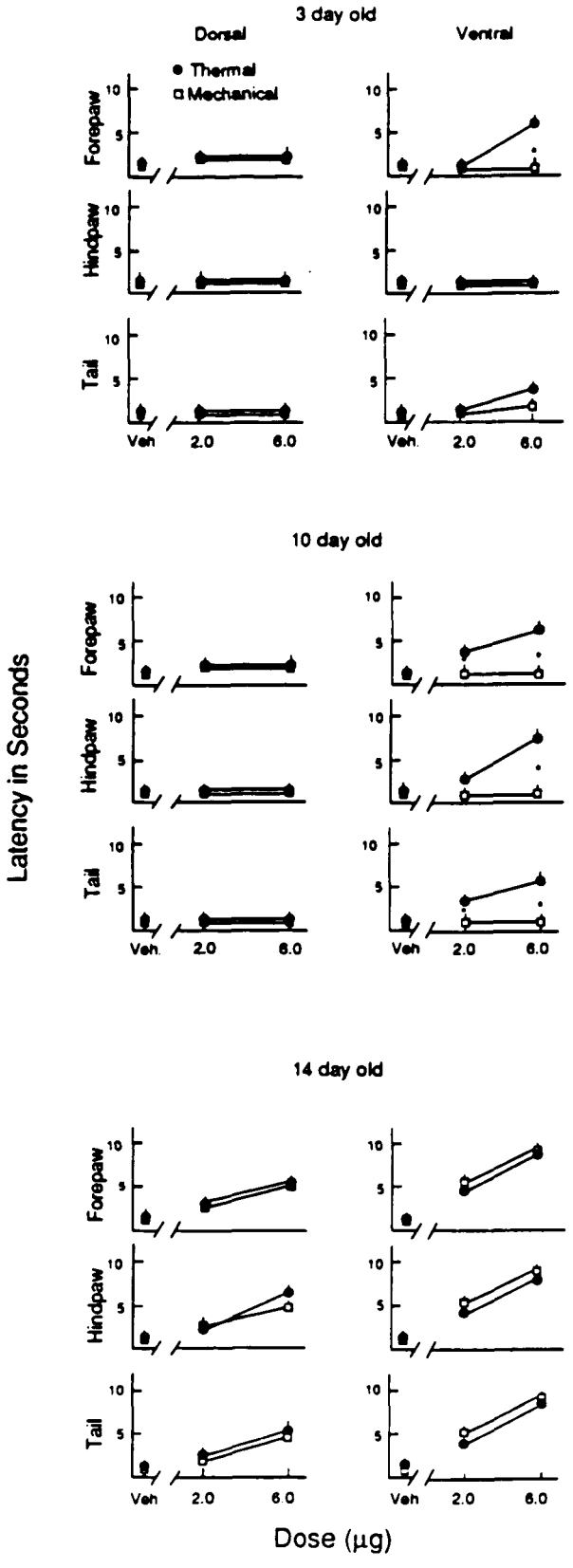

Vehicle
2 µg
6 µg
3 day old
10 day old
14 day old

Figure 2. Graphic representation of the effects of morphine administered to the dorsal and ventral PAG of rat pups aged 3, 10 and 14 days against thermal and mechanical noxious stimuli administered to the forepaw, hindpaw and tail. Closed circles represent withdrawal latencies from the thermal noxious stimulus while open squares represent withdrawal latencies from the mechanical noxious stimulus. Asterisks indicate a significant difference between two means.


3 day old
Dorsal
Ventral
Thermal
Mechanical
Forepaw
Hindpaw
Tail
10
5
Veh.
2.0
6.0
10 day old
14 day old
Latency in Seconds
Dose (µg)

Ventral sites The postnatal ontogeny of analgesia induced by morphine administration to the ventral PAG proceeded in a rostral to caudal direction. At 3 days of age, the high dose of morphine-induced analgesia against the thermal stimulus in the forepaw only. In the 10 day old pup, the high dose and the moderate dose of morphine induced analgesia against the thermal stimulus in the forepaw and only the high dose of morphine was sufficient to induce analgesia in the hindpaw and tail. In 14 day old pups, the moderate and high doses of morphine-induced antinociception against the thermal stimulus in all body parts in a dose dependent manner. Morphine administered to the ventral PAG did not induce analgesia against the mechanical stimulus until 14 days of age. At this age, however, analgesia of equal magnitude was seen against both types of stimuli in all body parts.

Dorsal sites In contrast to the early onset of morphine-induced analgesia mediated by the ventral PAG, morphine-induced analgesia against the thermal stimulus mediated by the dorsal PAG first emerged at 14 days of age. At this age, a significant level of analgesia against the thermal stimulus was induced by the moderate and high doses of morphine in a dose dependent manner in all body parts. Morphine-induced analgesia against the mechanical stimulus was also noted at 14 days of age. At this age, morphine administered to the dorsal PAG induced significant and dose dependent levels of analgesia against the mechanical stimulus in all body parts. At all ages tested in this experiment, the antinociception induced by morphine in the dorsal PAG was significantly less robust than the analgesia measured when morphine was given to the ventral PAG. The antinociceptive effects of morphine administered to the dorsal and ventral PAG of 3, 10 and 14 day old pups are displayed in Figure 2.

Glutamate

General Results: Glutamate was administered to 18 dorsal and 18 ventral sites at each of the three ages tested. The injection sites for glutamate are displayed in Figure 3. In 3 and 10 day old pups, glutamate-produced analgesia only when administered to the dorsal PAG. In addition, at these early ages glutamate-produced analgesia specifically against the mechanical noxious stimulus . In the 14 day old pup, glutamate-produced analgesia when given to either the dorsal or the ventral PAG and robust analgesia was seen against both thermal and mechanical noxious stimuli. These results are shown in Figure 4.

Figure 3. PAG sites to which glutamate was administered in 3, 10 and 14 day old rat pups.

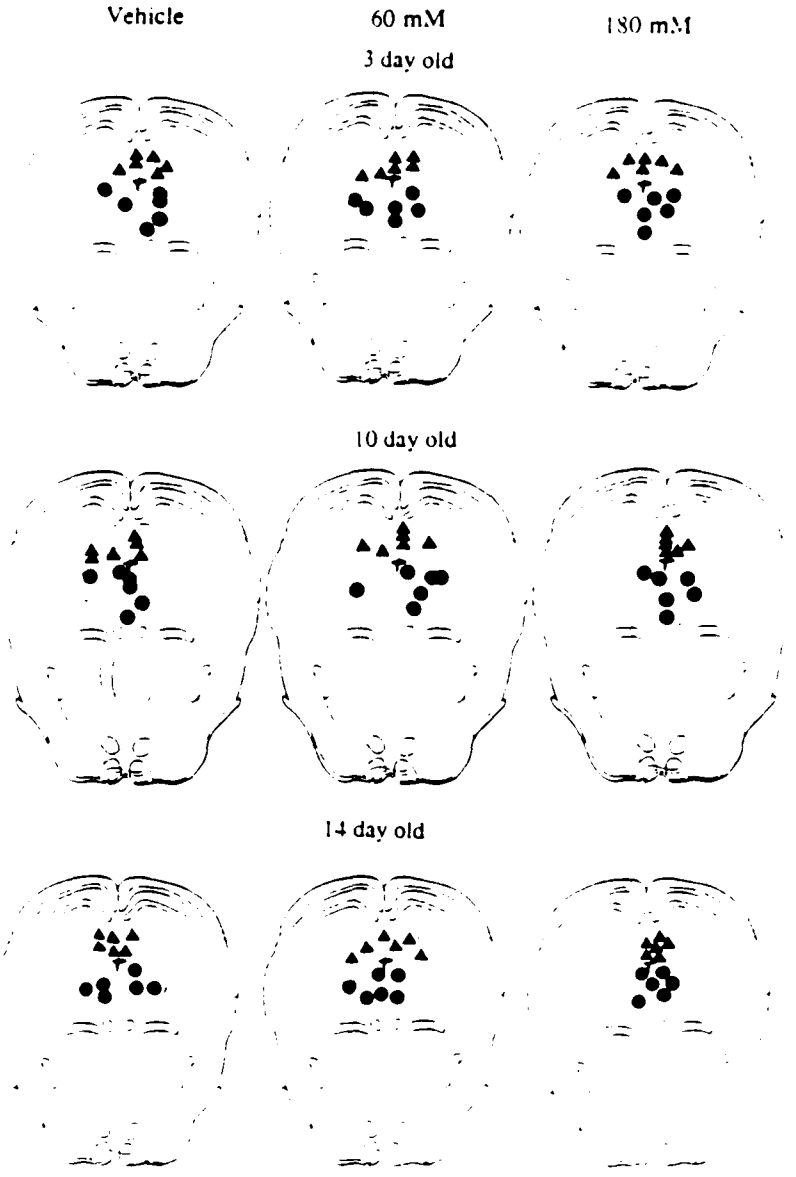
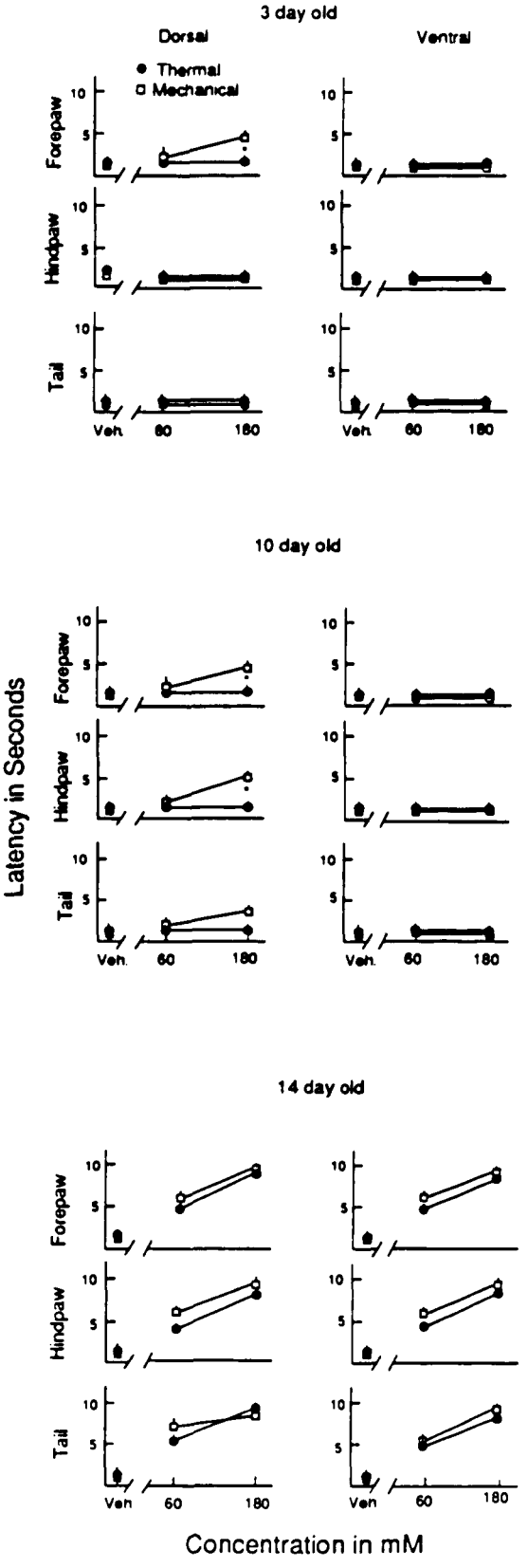

Vehicle
60 mM
180 mM
3 day old
10 day old
14 day old

Figure 4. Graphic representation of the analgesia produced by glutamate administration to the dorsal and ventral PAG of rat pups aged 3, 10 and 14 days against thermal and mechanical noxious stimuli administered to the forepaw, hindpaw and tail. Closed circles represent withdrawal latencies from the thermal noxious stimulus while open squares represent withdrawal latencies from the mechanical noxious stimulus. Asterisks indicate that there was a significant difference between two means.


3 day old
Dorsal
Ventral
Thermal
Mechanical
Forepaw
Hindpaw
Tail
10
5
Veh.
60
180
10 day old
14 day old
Latency in Seconds
Concentration in mM

Ventral sites The analgesic effects produced against the thermal stimulus by glutamate injections to ventral sites within the PAG emerged for the first time at 14 days of age. In 3 and 10 day old pups, glutamate administration to the ventral PAG did not produce significant analgesia against the thermal or mechanical stimulus in any of the body parts tested regardless of the concentration of glutamate given. In 14 day old pups, both the moderate and the high dose of glutamate administered to the ventral PAG produced robust and dose dependent analgesia against the both types of stimuli in the forepaw, hindpaw and tail.

Dorsal sites In 3 day old pups, the high concentration of glutamate administered to the dorsal PAG produced analgesia against the mechanical stimulus in the forepaw. In the 10 day old pup, the high concentration of glutamate-produced analgesia against the mechanical stimulus in the forepaw and hindpaw. In the 14 day old pup, potent and dose dependent analgesia was noted in the forepaw, hindpaw and tail in response to dorsal glutamate administration.When glutamate was administered to the dorsal PAG, analgesia against the thermal stimulus was produced for the first time at 14 days of age. At this age, the moderate and high doses of glutamate-produced dose dependent antinociception in the forepaw, hindpaw, and tail. In 3 and 10 day old pups, glutamate given to dorsal sites did not produce significant analgesia against the thermal stimulus in any body part tested even when the higher concentration of glutamate was given. A summary of the results of this experiment can be found in Table 3.

Table 3. Table 3 shows a summary of the results of Experiment 1. ++ signs indicate conditions under which there was a significant level of analgesia when the high and moderate doses of morphine or glutamate was given. + signs indicate conditions under which there was a significant level of analgesia when only the high dose of morphine or glutamate was given. - signs indicate conditions under which no analgesia was measured. **0** signs indicate conditions under which analgesia was measurable but did not reach significant levels (P values were greater than .05 but less than .07)

Morphine

	3 day Thermal	3 day Mechanical	10 day Thermal	10 day Mechanical	14 day Thermal	14 day Mechanical
Dorsal						
Forepaw	-	-	-	-	+	+
Hindpaw	-	-	-	-	+	+
Tail	-	-	-	-	+	+
Ventral						
Forepaw	+	-	++	-	++	+
Hindpaw	-	-	+	-	++	+
Tail	o	-	++	-	++	+

Glutamate

	3 day Thermal	3 day Mechanical	10 day Thermal	10 day Mechanical	14 day Thermal	14 day Mechanical
Dorsal						
Forepaw	-	+	-	+	++	++
Hindpaw	-	-	-	+	++	++
Tail	-	-	-	o	++	++
Ventral						
Forepaw	-	-	-	-	++	++
Hindpaw	-	-	-	-	++	++
Tail	-	-	-	-	++	++

Discussion

This study was the first to compare the analgesic effects of glutamate and morphine administration to the PAG of developing animals. The results indicate that the glutamate-produced and morphine-induced analgesia supported by the dorsal and ventral PAG can be distinguished developmentally. The dorsal PAG supported glutamate-produced analgesia at a very early age and morphine-induced analgesia for the first time at 14 days of age. In direct contrast to this, the ventral PAG supported morphine-induced analgesia early in development. Glutamate-produced analgesia is not supported by the ventral PAG until 14 days of age. In addition, the dorsal PAG supports analgesia against a mechanical stimulus exclusively at 3 and 10 days of age, while the ventral PAG supports analgesia against the thermal noxious stimulus at these ages. The site and stimulus differences evinced in the production of analgesia by these compounds during development indicate that these compounds utilize different neural systems either within, or external to, the PAG in order to produce analgesia during development and possibly in the adult animal as well.

In this study, SPA in the PAG was elicited using glutamate rather than the focal electrical stimulation. There were several reasons for using glutamate. It was the goal of this study to distinguish the antinociceptive effects of opiate-induced analgesia from those of stimulation-produced analgesia in developing animals. When making such a comparison, it is important to keep as many factors as possible constant. One advantage gained from using glutamate in this experiment was that the effects of glutamate, like those of morphine, are receptor mediated. The effects of electrical stimulation are not receptor mediated. In addition, glutamate focally stimulates cell bodies and does not excite neurons of passage as does electrical stimulation (Goodchild, et al., 1982). Moreover, the potential for unintentional orthodromic stimulation of neighboring neurons involved in nociception is eliminated by the use of glutaminergic stimulation rather than electrical stimulation.

Yaksh and Jensen (1989) compared the analgesic effects of glutamate and morphine at identical sites within the PAG in adult rats. They found that both compounds produced the same effect in all areas of the PAG. In the present study, we found that glutamate-produced identical analgesic effects in the 14 day old rat pup, regardless of the site of injection within the PAG. However, morphine produced consistently lower levels of analgesia when administered to the dorsal PAG than when administered to the ventral PAG. It is possible that the differences between our findings and theirs may be attributable to the doses of morphine given. The doses of morphine sulphate used in their experiment

were 10-30 µg/ 0.5 µl while the doses of morphine sulphate used in our experiment were 2-6 µg/0.2 µl. It is also possible that the dorsal and ventral PAG distinctions that are seen even in the 14 day old pup with regard to morphine analgesia may disappear completely in the fully mature animal due to further development of receptor systems.within or efferent to the PAG.

Several groups have examined the analgesic effects of morphine administration to, and electrical stimulation of the PAG, and some have compared the effects of opiate analgesia and electrical SPA at coincident sites within the PAG. In 1976, Yaksh et al. found that morphine elicited potent, whole body, analgesia when administered to the caudal aspect of the PAG. Morphine administration to rostral PAG regions produced analgesia that was limited to more rostral body parts of the rat. The sites that are best for supporting electrical SPA within the PAG have also been determined. Fardin et al. (1984) found that the best sites for obtaining "pure" SPA (unaccompanied by motor side effects or vocalization) were located in the dorsomedial aspect of the DRN found embedded within the caudal PAG, as well as within the ventrolateral PAG. Nichols et al. (1989) found that sites caudal to the DRN did not support analgesia at all. In 1977, Lewis and Gebhart compared the antinociceptive effects of morphine and electrical stimulation at concurrent PAG sites. They found that the optimal sites for opiate analgesia and SPA within the PAG were not the same. Morphine was more effective at producing analgesia when applied medially within the PAG, whereas stimulation was most effective when given to the ventrolateral edge of the PAG. This group also found (1976) that the analgesia produced by morphine administration to the PAG was not duplicated by other centrally acting agents such as chlorpromazine, chlordiapoxide, pentobarbital, or naloxone when administered there. Therefore, there is a site specificity for morphine within the PAG. Yeung et al. (1977) determined that although sites sensitive to morphine were restricted to regions inside the boundaries of the PAG, sites sensitive to SPA extended to regions peripheral to the PAG as well. The fact that several laboratories have determined that the optimal sites within the PAG for OA and SPA differ from each other supports the hypothesis that these forms of analgesia are differentially mediated by the PAG. In the present study, care was taken to implant cannulae into the caudal aspect of the PAG, at the level of the DRN, as this area appeared to be an effective site for supporting both opiate analgesia and electrical SPA in all of the aforementioned studies.

Some of the ventral implant sites in the present study actually fell within the borders of the DRN. Stimulation of DRN sites with morphine or glutamate resulted in analgesia that was

statistically indistinguishable from analgesia obtained from other ventral PAG sites. Therefore implants falling within the DRN were considered ventral PAG sites in this study. Oliveras et al. (1974) proposed that the ventral region of the PAG is the only region to support true analgesia. Stimulation of the dorsal region of the PAG produced aversive responding, avoidance behavior and vocalizations in addition to analgesia. Therefore, the analgesic effect of dorsal PAG stimulation was hypothesized to be be secondary to these other behaviors. However, benzodiazepines administered systemically (Morgan et al., 1987), or to the PAG (Leao Borges et al., 1988) attenuated the aversive effects of dorsal PAG stimulation without effecting the analgesia produced by that stimulation. These studies provided evidence for a distinction between the analgesic effects of dorsal PAG stimulation and the aversive behaviors that accompany such stimulation. The results of the present study support this distinction. Analgesia, unaccompanied by aversive reactions, was noted following glutaminergic stimulation of the dorsal PAG in pups at 3, 10 and 14 days of age and following morphine stimulation of this area in 14 day old pups. It was not until 21 days of age (unpublished observations) that stimulation of sites within the dorsal PAG produced aversive responses together with analgesia. Thus, the analgesic and aversive behaviors following dorsal PAG stimulation can be distinguished both developmentally and pharmacologically.

Both morphine-induced and stimulation-produced analgesia mediated by the PAG developed in a rostral to caudal direction. In general, the analgesia produced by both drugs was first noted in the forepaw at 3 days of age and was not seen in the hindpaw or tail until 10 days of age. These findings concur with those of Giordano and Barr (1987), who found that systemic morphine-induced analgesia in the forepaw early in development, and that these analgesic effects did not extend to the hindpaw and tail until later in development. It is likely that the rostral to caudal development of morphine-induced and glutamate-produced analgesia mirrors the growth of descending bulbospinal neurons originating in the RVM that are excited or disinhibited by the administration of analgesia producing compounds administered to the PAG. These spinopetal systems do not develop uniformly (Bregman, 1987; Simmons and Jones et al.,1985; Commisiong, 1983) and may differentially modulate analgesia against thermal and mechanical nociceptive stimuli (Kuraishi et al., 1983 and 1985; Satoh et al., 1983; Hughes and Barr, 1988; Giordano and Barr, 1988). One exception to this was noted when morphine was given to the ventral PAG of 3 day old rats. In this instance, analgesia was noted in the tail but not the hindpaw. This analgesia did not reach significant levels, yet it was noteworthy and conflicts with the model that analgesia from the PAG is mediated by bulbospinal systems

that develop in a rostral to caudal direction. Studies in our laboratory (Paredes and Barr, unpublished observations) revealed that the tail withdrawal response is spinally mediaded whereas the hindpaw withdrawal response is supraspinally mediated. Therefore, it is possible that projections from the RVM to the spinal cord that mediate analgesia from the ventral PAG are almost fully developed in the neonatal rat pup. However, the hindpaw withdrawal response might be more dependent upon the maturation of ascending projections than on mature descending bulbospinal projections. In addition, spinal innervation of the hindpaw and the tail is very different. Differences in the development of spinal projections to each area might also account for the fact that analgesia is seen earlier in the tail than in the hindpaw in some instances. Nonetheless, in most cases the rostral to caudal development and the stimulus specificity of the analgesia produced by PAG stimulation at early ages probably reflects developmental changes within these bulbospinal systems.

The differences between analgesia supported by the dorsal and ventral PAG that were noted in the present experiment are in accordance with the findings of others (Morgan and Liebeskind, 1987; Prieto et al., 1983; Behbehani and Fields, 1983; Cannon et al., 1982; Nichols et al., 1989). These groups also found that stimulation of the dorsal PAG, either electrically or chemically, elicited antinociceptive effects that were distinct in two ways from those supported by the ventral PAG. The first was the facility with which this analgesia could be antagonized by naloxone. The second was the differential development of cross tolerance to morphine-induced analgesia. The ventral PAG supported an opioid form of SPA that was antagonized by naloxone and cross tolerant to the effects of morphine, whereas the dorsal PAG supported a non-opioid form of analgesia. In the present study, glutamate-produced analgesia was supported at an earlier developmental stage by the dorsal PAG than the ventral PAG, while the converse was true for morphine-induced analgesia. This indicates that the substrates that underlie GPA are present exclusively in the dorsal PAG of very young rat pups, while the substrates that support opiate analgesia are localized exclusively in the ventral PAG of neonatal rats.

The differential analgesic effects produced by morphine or glutamate administration to the PAG during development may be due to the differential pharmacological action of these compounds in both neonatal and adult animals. Recent evidence suggested that opiates administered to the PAG may induce analgesia by inhibiting inhibitory interneurons that utilize the inhibitory amino acid neurotransmitter γ-Aminobutyric acid (GABA). The PAG has been shown to be rich in GABA (Barbaresi and Manfrini, 1988). The amino acid is

probably contained within intrinsic neurons, as the deafferented brain does not show major changes in GABA activity (Sandner et al.,1981). Recently, GABA has been localized in neurons and terminals within the PAG using immunohistochemistry (Barbaresi et al., 1988). In addition to being present within the PAG, GABAergic activity seems to contribute to the analgesia that is supported by the PAG. The GABA antagonists picrotoxin and bicuculline produce effects similar to those of morphine when administered to the PAG (Moreau and Fields, 1986). Microinjection of these compounds into the PAG increased tail flick latencies and increased the firing of cells in the RVM. The common effects of morphine and these GABA antagonists are antagonized by the administration of the $GABA_A$ agonist muscimol to the PAG, indicating that there is a link between opiate analgesia and the $GABA_A$ receptor within the PAG. Furthermore, bicuculline administration to the PAG has been shown to abolish noxious heat evoked activity of cells in the dorsal horn of the spinal cord to the same degree that morphine does (Sandkuhler et al., 1989). Thus, morphine administered to the PAG may induce analgesia indirectly by inhibiting the tonic inhibition that GABAergic cells would normally exert on a descending antinociceptive pathway. Opiates have been shown to excite pyramidal cells within the hippocampus by inhibiting adjacent inhibitory neurons there. It is possible that opiates might play a similar role within the PAG.

In this experiment, the high dose of morphine did not produce cutoff levels of analgesia when given to dorsal PAG sites in 14 day old pups. Data that are not presented showed that morphine givent to the dorsal PAG of 21 day old pups still did not produce total analgesia. Therefore, it is quite likely that morphine interacts with distinct opioid receptor populations when administered to the dorsal and ventral PAG. Binding studies have shown that μ opiate receptors are more numerous in ventral than dorsal regions of the PAG. Therefore, morphine probably interacts with a unique population of opioid receptors in the dorsal PAG and may be a partial agonist at those receptors. A good candidate for this receptor type is the κ_2 recently described by Gistrak et al.(1989). These receptors act as partial agonists for morphine supraspinally and may be involved in mediating the effects of morphine administered to the dorsal PAG.

In contrast to the indirect activation of descending bulbospinal systems thought to be induced by morphine administration to the PAG, glutamate administration to the PAG is thought to activate these descending bulbospinal systems directly. The glutamate receptor agonist NMDA produced potent analgesia when administered to the PAG This analgesia was not reversed by naloxone, indicating that the analgesia produced by NMDA is

independent of opioid activity (Jaquet, 1988). However, the NMDA receptor antagonist D-AP7 attenuated both NMDA produced and morphine-induced analgesia supported by the PAG indicating some overlap between the analgesia mediated by glutamate and morphine in the PAG (Jaquet, 1988). The analgesic effects of NMDA and morphine administered to the PAG appeared to be additive. Naloxone caused the analgesic effects of both compounds to return to that of NMDA alone (Jaquet, 1988). Thus, different neural substrates acted upon by glutamate and morphine administered to the PAG may contribute to the different analgesic effects of these compounds in the developing animal. At present, there is very little data regarding the development of GABAergic systems and glutaminergic systems within the PAG. However, it is certainly possible that these inhibitory and excitatory systems may develop at different rates within the dorsal and ventral aspects of the PAG, and that the differential ontogeny of these systems could contribute to the differences in analgesia supported by these regions of the PAG in the developing animal.

Experiment 2

The aim of this experiment was to compare the effects of systemic naloxone on the analgesia produced by glutamate and morphine administered to sites within the dorsal and ventral PAG in developing animals. In Experiment 1, it was shown that glutamate-produced and morphine-induced analgesia mediated by the PAG do not develop at the same rate. These findings indicate that the analgesia produced by glutamate or morphine administration to the PAG are either supported by distinct substrates within the PAG, or that they differentially activate efferent systems that originate in the PAG and project to the RVM, or both. The present study attempts to determine whether glutamate and morphine both activate an opioid system and whether the dorsal and ventral subdivisions of the PAG differentially support opioid and non-opioid forms of analgesia in developing animals, as they appear to in adult animals

A traditional way to determine whether an antinociceptive system is opioid in nature is to attempt to reverse the analgesia produced by that system with the opiate antagonist naloxone. The analgesic effects of morphine (Kuraishi et al, 1978; Yaksh and Tyce, 1979), electrical stimulation (Akil et al., 1976; Cannon et al., 1982; Nichols et al., 1989) and glutamate (Urca et al., 1980) applied to the PAG or the RVM were reversed by systemic administration of naloxone. Fields and Basbaum (1984) suggested that

stimulation of, or morphine microinjection into, the PAG or RVM nuclei might activate descending monoaminergic neurons from the RVM that synapse on enkephalinergic interneurons in the dorsal horn. According to their model, the release of 5-HT and NE by these spinopetal neurons may stimulate spinal interneurons to release enkephalins. These enkephalins may in turn bind to opiate receptors within the dorsal horn and induce analgesia by acting on the same neural substrates as are involved in analgesia induced by intrathecal opiate administration. Thus, the ability of systemic naloxone to attenuate analgesia mediated by the PAG or NRM could be due to naloxone antagonism of spinal opiate receptors. This hypothesis was supported by the work of Zorman et al., (1981 and 1982) who found that the analgesia produced in response to electrical microstimulation of the NRM and the NRPG was reversed by both systemic and intrathecal naloxone administration. This model has been challenged by the fact that some laboratories reported that the antinociceptive effects of opiate administration to (Jensen and Yaksh, 1986b) or electrical (Aimone et al., 1987) or glutaminergic (Jensen and Yaksh, 1984; Satoh et al., 1983) stimulation of, the PAG or sites within the RVM were not attenuated by intrathecal naloxone administration. In addition, Jensen and Yaksh (1986b) found that the analgesic effects of morphine applied to the NRM and the NRGC could be reversed by naloxone administration to the PAG, but the effects of morphine administered to the PAG could not be reversed by naloxone administration to these RVM nuclei. Thus, the analgesia produced in response to PAG stimulation is mediated by opiate receptors within the PAG, and does not appear to involve the activation of spinal opiate receptors. Therefore, when analgesia supported by the PAG is reversed by systemic naloxone, this reversal can be said to be caused by the blockade of opiate receptors within the PAG.

Systemic naloxone has been consistantly shown to reverse the analgesic effects of morphine given systemically or focally to brainstem nuclei. However, the effects of naloxone on electrical or glutaminergic stimulation-produced analgesia are much less clear. Glutamate-produced analgesia mediated by the PAG was either reversed or potentiated by systemic naloxone pretreatment depending on the time that the naloxone was given relative to the glutamate administration and the dose of naloxone given (Urca et al., 1980). The analgesic effects of glutamate stimulation of the PAG were not reversed by intrathecal administration of naloxone (Jensen and Yaksh, 1984). However, the analgesia produced by glutaminergic stimulation of the RVM has been shown both to be reversed (Jensen and Yaksh, 1984) or not reversed (Satoh, et al., 1983) by intrathecal naloxone treatment. Electrical SPA arising in the PAG was reversed (Oliveras et al. 1977; Cannon et al., 1982;

Nichols et al.,1989), partially reversed (Akil et al., 1976), or not reversed at all (Yaksh et al., 1976; Klatt et al., 1988) by systemic naloxone depending upon what portion of the PAG was stimulated. Naloxone, administered systemically or to the PAG, elevated thresholds for PAG stimulation at ventral but not dorsal sites within the PAG (Yeung et al,1977; Lewis and Gebhart, 1977; Cannon et al., 1982; Nichols et al. 1989). This finding provided a possible explanation for the conflicting results previously obtained. The PAG was divided into two discrete regions based on the results of naloxone studies: A dorsal region that supports SPA and GPA that are not naloxone reversible, and a ventral region that supports OA, SPA and GPA that are naloxone reversible. It is possible that SPA supported by the ventral region of the PAG shares a common mechanism of action with morphine, while SPA supported by the dorsal PAG may have a distinct, and as yet not clearly understood, mechanism of action. The goal of this study was to test the hypothesis that naloxone differentially antagonizes analgesia supported by the dorsal and ventral PAG by comparing the effect of systemic naloxone on morphine-induced and glutamate-produced analgesia supported by the dorsal and ventral PAG in developing animals.

Methods

Naloxone treatment

Ten and 14 day old pups were used in this experiment. Two pups from a single litter were implanted with cannulae aimed at the PAG as previously described. Pairs of sibling 10 day old pups were given one of two treatments: morphine administered to the ventral PAG or glutamate administered to the dorsal PAG . This was done because in the 10 day old pup, the ventral PAG was found to be the only effective site for inducing morphine analgesia while the dorsal PAG was found to be the only effective site for producing glutamate analgesia. Pairs of 14 day old siblings were given either glutamate or morphine injections to the dorsal or the ventral PAG. This was done because in the 14 day old rat pup, both glutamate and morphine were found to produce analgesia when administered to either dorsal or ventral PAG sites. Pups were given an intraperitoneal (i.p.) injection of 1 mg/kg of naloxone or of the saline vehicle. Five minutes later, 180mM of glutamate was administered to the dorsal PAG or 6 μg of morphine was given to the ventral PAG of both pups. Analgesia was measured against the thermal and mechanical stimulus as described previously.

Statistics

Data for each treatment given to 14 day old pups were analyzed using a five way analysis of variance. Between litter factors were PAG site (dorsal or ventral) and intraPAG drug treatment (glutamate or morphine). Within litter factors were intraperitoneal treatment (naloxone or saline), stimulus type (thermal or mechanical) and body part (forepaw, hindpaw or tail). Post hoc Tukey tests were done to ascertain the nature of significant interactions.

Results

The results of the five analysis of variance for this experiment are shown in Table 4.

Table 4. The results of the analysis of variance for Experiment 2 are shown in tabular form. Abbreviations are as follows: P is PAG site (Dorsal or Ventral), C is intraPAG compound (Morphine or Glutamate), S is stimulus type (Thermal or Mechanical), L is location on the body (Forepaw, Hindpaw or Tail), T is intraperitoneal treatment (Naloxone or Saline).

Source	Sum of Squares	Degrees of Freedom	Mean Square	F	Tail Probability
P	2550.00	2	1225.00	1309.00	0.0000
C	501.73	1	404.23	100.00	0.0000
PC	300.32	2	155.60	164.65	0.0000
S	200.56	1	200.56	80.34	0.0005
SP	144.24	2	72.12	54.62	0.0651
SC	298.92	1	298.92	105.75	0.0582
SPC	120.24	2	60.14	18.90	0.0715
L	6.87	1	6.87	11.95	0.0346
LP	24.30	2	12.15	25.09	0.0256
LC	75.90	1	75.90	135.62	0.0629
LPC	15.80	2	7.90	10.98	0.1247
SL	25.07	1	25.07	80.98	0.0000
SLP	28.06	2	14.03	51.24	0.0000
SLC	8.88	1	8.88	30.27	0.0000
SLPC	17.08	2	8.54	29.75	0.0023
T	1488.42	2	744.21	599.02	0.0000
TP	1204.24	4	301.06	234.98	0.0000
TC	54.72	2	27.36	22.78	0.0000
TPC	104.20	4	31.10	19.80	0.0001
TL	125.54	2	62.77	41.85	0.0345
TLP	118.26	4	39.24	26.89	0.0267
TLC	180.06	2	90.03	56.43	0.0321
TLPC	40.20	4	10.05	6.63	0.0479
TS	4.06	2	2.03	4.06	0.0000
TSP	10.62	4	2.65	5.57	0.0000
TSC	42.97	2	21.48	43.27	0.0000
TSPC	9.36	4	2.34	6.07	0.0007
TLS	13.87	2	6.50	13.92	0.0125
TLSP	20.8	4	5.20	8.95	0.0233
TLSC	3.52	2	1.76	4.32	0.0453
TLSPC	12.44	4	3.11	5.08	0.0452

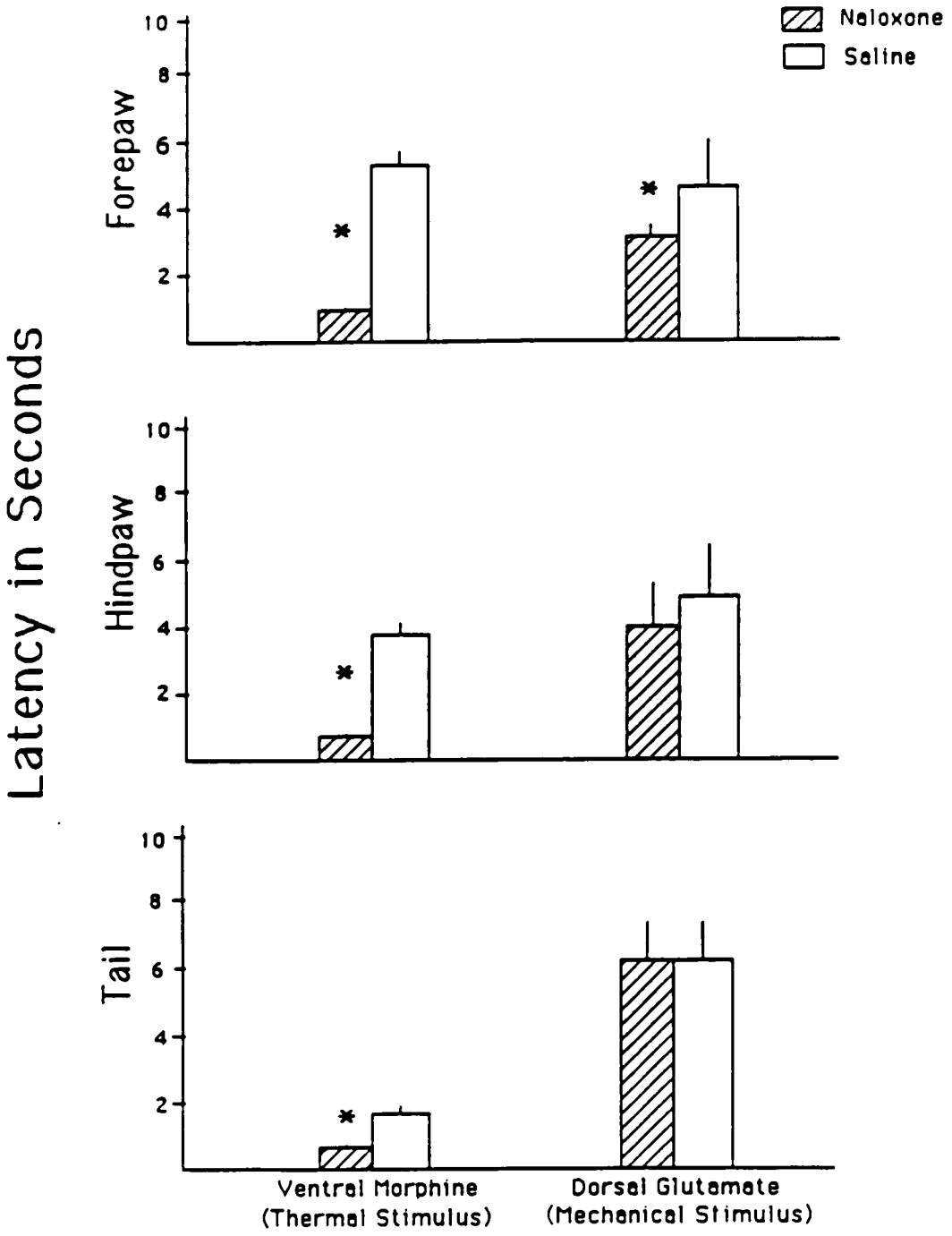
Ten day old pups

Naloxone significantly blocked analgesia induced by morphine administered to the ventral aspect of the PAG. In general, naloxone did not attenuate analgesia produced in response to glutamate administration to the dorsal PAG. However, the analgesia produced in the forepaw in response to glutamate given to the dorsal PAG was reversed significantly by naloxone. These results are shown in Figure 5.

Fourteen day old pups

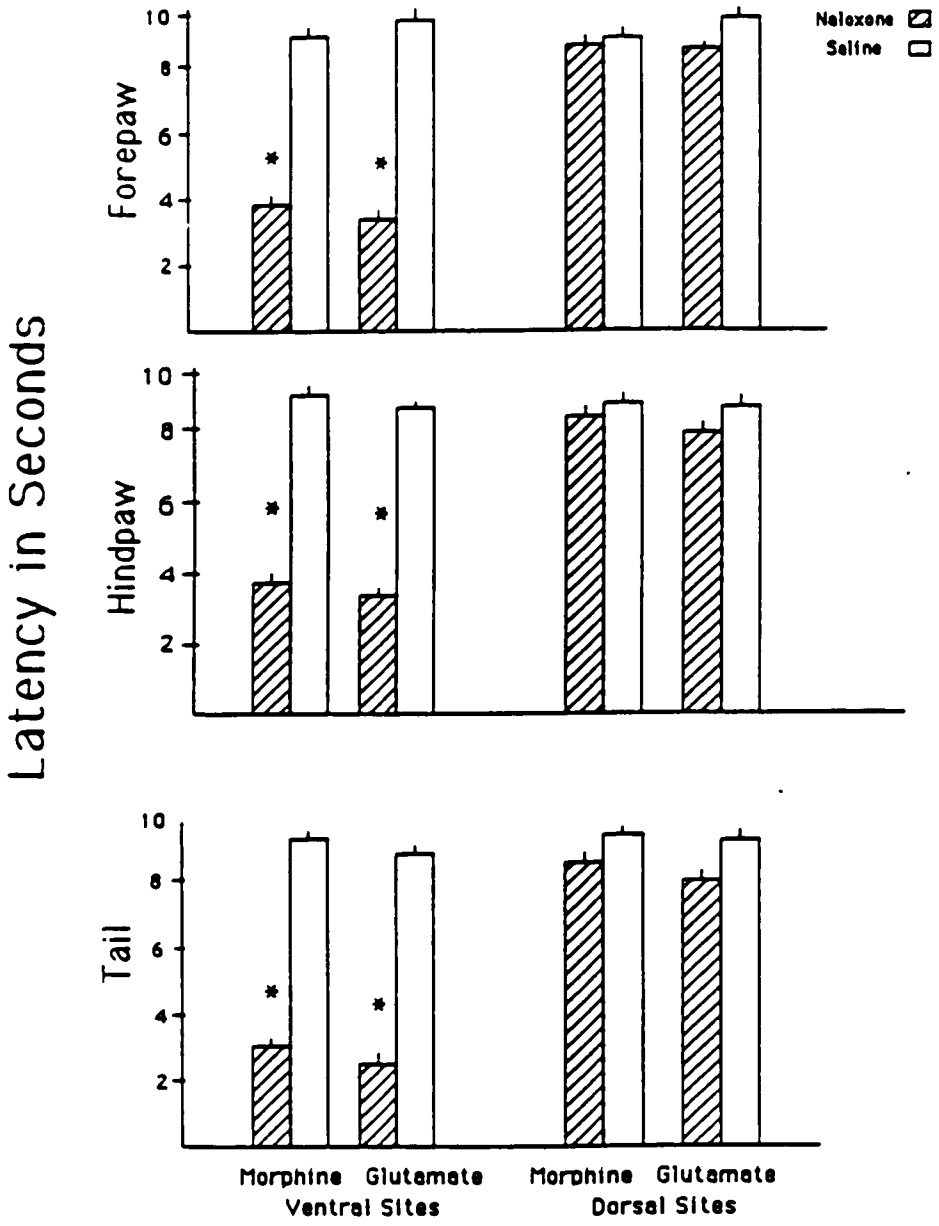
Naloxone significantly antagonized the antinociceptive effects of both glutamate and morphine at all body sites when they were administered to the ventral aspect of the PAG. However, naloxone did not block the antinociception produced by either compound given to the dorsal PAG. These data were collapsed over stimulus type because no significant stimulus differences were noted in the analgesia resulting from administration of the high dose of morphine or glutamate to the PAG of 14 day old pups. Figure 6 shows the effects of systemic naloxone pretreatment on morphine-induced and glutamate-produced analgesia mediated by the dorsal and ventral PAG in 14 day old pups.
A summary of the data for 10 day old and 14 day old pups are shown in Table 5.

Figure 5. This graph shows the comparative effects of naloxone and saline control on morphine-induced analgesia supported by the ventral PAG or glutamate-produced analgesia supported by the dorsal PAG in 10 day old rat pups. Latencies for withdrawal of the forepaw, hindpaw and tail from thermal or mechanical stimuli are shown. Striped bars represent withdrawal latencies after pretreatment of intraperitoneal naloxone administration followed by morphine or glutamate. Solid bars represent withdrawal latencies following pretreatment of naloxone control followed by morphine or glutamate. Asterisks indicate significance. Vertical lines indicate standard errors of the mean.



Effects of Naloxone on Morphine-Induced and Glutamate-Produced Analgesia in 10 day old rats.

Figure 6. This graph shows the comparative effects of naloxone and saline control on morphine-induced analgesia and glutamate-produced analgesia mediated by dorsal and ventral PAG sites in 14 day old rat pups. Latencies for withdrawal of the forepaw, hindpaw and tail from thermal or mechanical stimuli are shown. Striped bars represent withdrawal latencies following intraperitoneal naloxone administration and solid bars represent withdrawal latencies fo lowing administration of naloxone control. Asterisks indicate significance. Vertical lines indicate standard errors of the mean.



Effect of Naloxone Pretreatment on 14 day old rat pups

Table 5. The results of Experiment 2 are summarized in tabular form. The + signs indicate conditions under which 1 mg/kg naloxone administered intraperitioneally significantly reversed morphine-induced or glutamate produced analgesia. The - signs indicate conditions under which naloxone did not anatagonize morphine or glutamate analgesia.

10 day old

Morphine 6 μg Ventral	Glutamate 180 mM Dorsal
+	+
+	-
+	-

14 day old

Morphine 6 μg

Dorsal	Ventral
+	-
+	-
+	-

Glutamate 180 mM

Morphine 6 μg	180 mM Dorsal
+	-
+	-
+	-

Discussion

The results of this study showed that systemic naloxone reversed both morphine-induced analgesia and glutamate SPA mediated by the ventral PAG, but did not have any effect on analgesia supported by dorsal PAG sites during development. These findings support the premise of a dorsal-ventral distinction within the PAG. This dichotomy was also found by Cannon et al. (1982) and more recently by Nichols et al. (1989), both of whom used electrical stimulation of the PAG to produce analgesia in adult animals. Urca et al. (1980) and Jensen and Yaksh (1984) have examined effects of naloxone on glutamate-produced analgesia mediated by the PAG in adult animals. They did not note a dorsal-ventral PAG distinction with regard to the effects of naloxone. However, they did not publish the location of their implant sites within the PAG. Therefore, it is possible that all or many glutamate injections were made to the ventral PAG in their studies.

There appear to be two types of projections between cells in the PAG and cells in the RVM. Autoradiographic, electrophysiological, pharmacological and behavioral evidence suggest that some projections from the PAG to the RVM utilize an excitatory neurotransmitter. Autoradiographic labelling using D-^{3}H aspartate, a label that is relatively specific for excitatory amino acids, has demonstrated probable excitatory projections from the PAG to the NRM. The existence of these excitatory projections has been supported by electrophysiological evidence. Electrical stimulation of cells in the ventrolateral PAG produced a resultant excitation of cells in the NRM that was reversed by the generic excitatory amino acid antagonists g-D-glutamyl-glycine (DGG) or kynurenate, but not by the specific NMDA receptor antagonists ketamine or amino-5-phosphonoheptanoate (Wilkund et al., 1988). Pharmacological data has also confirmed an excitatory link between the PAG and the NRM. Under normal conditions many compounds, when injected into the NRM, have been shown to antagonize analgesia produced by electrical stimulation of the PAG. These include the opiate antagonist naloxone, the serotonin antagonist methysergide, the anesthetic lidocaine and the exitatory amino acid antagonists DGG and DL-2-amino-5-phosphonovalerate (APV). However, when the function of neurons in NRPG and NRGC was blocked with lidocaine, the excitatory amino acid antagonists DGG and APV were the only antagonists that continued to attenuate the excitation of NRM neurons produced by PAG stimulation (Aimone and Gebhart, 1986). This indicates that there is an excitatory link between the PAG and the NRM that is activated when cells within the PAG are stimulated electrically. In addition, administration of L-glutamate to the NRM and NRPG has been shown to produce potent analgesia against

noxious tail pinch and heat applied to the tail (Satoh et al., 1982 and Jensen and Yaksh, 1988). These findings confirm the existance of an excitatory connection between the PAG and the RVM nuclei that modulate nociceptive responding. It is thought that this excitatory pathway is activated when cells in the PAG are stimulated electrically or by the administration of morphine or glutamate.

There is also evidence for an enkephalinergic link between the PAG and these medullary nuclei. Enkephalinergic cell bodies and terminals have been localized in the NRM, NRGC, and NRPG as well as the in the PAG (Hokfelt, 1977). Administration of morphine to the NRM, NRGC or the NRPG produces potent behavioral analgesia (Dickenson et al, 1979; Kuraishi et al.,1979 ; Takagi et al, 1976 and 1977; Levy et al 1979, Azami et al, 1982; Satoh et al., 1979) that is reversible by naloxone administration to the RVM nuclei and to the PAG (Jensen and Yaksh, 1986b). Morphine and met-enkephalin microinjections into some of these RVM nuclei has been associated with an increase in normetanephrine (Kuraishi et al., 1978), norepinephrine, and serotonin (Hammond et al., 1985) levels in the spinal cord. These findings indicate that there is most probably an enkephalinergic link between the PAG and the RVM nuclei and that this link is involved in the bulbospinal analgesic system previously described.

A possible explanation for the differential ability of sites within the dorsal and ventral PAG to support opiate and non-opiate analgesia may therefore be due to the fact that projections from the dorsal PAG to RVM nuclei may be predominantly glutaminergic, while projections from the ventral PAG to sites within the RVM might be predominantly enkephalinergic. This hypothesis would account for the fact that systemic naloxone reverses analgesia from ventral, but not dorsal sites within the PAG, but does not account for the failure of naloxone to reverse morphine-induced analgesia supported by the dorsal PAG. However, the attenuation of the analgesic effects of morphine administered to the ventral PAG by systemic naloxone might be due to the blockade of RVM opiate receptor sites that are necessary for the production of analgesia from ventral, but not from dorsal sites within the PAG. Glutamate stimulation of cells in the RVM might directly excite a spinal monoaminergic pathway, while enkephalin release into these nuclei might inhibit tonic inhibitory influences on this pathway. This theory is strengthened by the fact that intrathecal naloxone was shown to have little effect on morphine or glutamate analgesia mediated by the PAG. Thus, the opioid link for supraspinally produced analgesia may reside at the medullary level rather than at the spinal level.

Experiment 3

Several lines of evidence support the premise that morphine-induced analgesia is modulated by spinal 5-HT and NE. Analgesia induced by morphine injections into the NRM is antagonized by intrathecal injections of the 5-HT antagonist methysergide, but not by the a NE antagonist phentolamine (Jensen and Yaksh, 1986). Conversely, microinjections of morphine into the NRGC produced analgesia that was reversed to a greater extent by phentolamine than by methysergide (Jensen and Yaksh, 1986). Furthermore, iontophoretic administration of 5-HT and NE to the substantia gelatinosa of the spinal cord inhibited the discharge of nociceptive neurons that was reversed by monoamine antagonists but not by naloxone (Belcher et al., 1978, Headley et al., 1978). Intrathecal administration of 5-HT or α NE antagonists, in the absence of morphine, elevated nociceptive thresholds in several species ranging from rat to primate (Reddy and Yaksh, 1980; Reddy et al., 1980; Yaksh and Wilson,1979, Hughes and Barr,1988). Morphine administration to the PAG caused increased turnover of 5-HT (Shiomi et al., 1978) and NE (Shiomi and Takagi, 1974) in the spinal cord. Injections of morphine into the PAG caused the release of 5-HT from the spinal cord while morphine microinjections into the NRM have been shown to increase rates of 5-HT synthesis in the spinal cord (Yaksh and Tyce, 1979).

Jensen and Yaksh (1986) compared the analgesic effects of morphine administered to the PAG, the NRM, and the NRGC. They found that potent analgesia was induced following morphine administration to all three injections sites against the supraspinal hot plate response (HP). The administration of morphine to the PAG produced full attenuation of the spinally mediated tail flick reflex (TF). When morphine was given to the NRM and the NRGC analgesia assessed by the TF showed a clear plateau even when the dose of morphine was increased threefold and when bilateral injections of morphine were given to the NRGC. These results indicated that the PAG, the NRM and the NRGC are all involved in the production of morphine-induced analgesia, but that they have different roles in the production of this analgesia. Moreover, the antinociceptive effects of morphine injected into the PAG on the TF, but not the HP, were fully antagonized by a combination of the serotonergic antagonist methysergide and the noradrenergic antagonist phentolamine administered intrathecally, but neither of these treatments alone was sufficient to fully antagonize the TF analgesia produced by the administration of morphine to the PAG (Yaksh, 1979). Jensen and Yaksh (1986) combined microinjections of morphine to the PAG, NRM or NRGC with intrathecal injections of serotonergic (methysergide),

noradrenergic (phentolamine), opiate (naloxone) and dopaminergic (cis-flupenthixol) antagonists and assessed the degree to which these intrathecal treatments attenuated morphine-induced analgesia. They found that the TF inhibition induced by the microinjection of morphine into the PAG was fully antagonized only by administering a combination of methysergide and phentolamine intrathecally. No other treatment was sufficient to fully antagonize morphine-induced analgesia mediated by the PAG. The TF inhibition produced by the administration of morphine to the NRGC was antagonized most effectvely by intrathecal phentolamine, less effectively by intrathecal naloxone, and not at all by intrathecal methysergide or cis-flupenthixol. In contrast to this, TF inhibition induced by morphine administration to the NRM was antagonized fully by intrathecal methysergide, partially by intrathecal naloxone, and not at all by intrathecal phentolamine or cis-flupenthixol. These experiments provide evidence that morphine administered to the PAG or the NRM causes 5-HT release from the spinal cord, whereas morphine administration to the PAG or the NRGC results in NE release from the spinal cord. These findings have been confirmed by other laboratories. Kuraishi et al. (1979) found that morphine microinjections to the NRGC were antagonized by systemic pretreatment with phenoxybenzamine (an α_1 NE antagonist) but not propanolol (a β adrenergic antagonist) or methysergide. This group also found (1978) that the microinjection of morphine or methionine-enkephalin into the NRGC produced a naloxone reversible increase in normetanephrine in the spinal cord. Zhong et al.(1985) found that intrathecal administration of DSP4, a highly selective noradrenergic neurotoxin, significantly attenuated morphine-induced analgesia whether morphine had been administered systemically or intraventricularly.

Analgesia produced by electrical stimulation of the PAG (SPA) appears to be modulated by the same spinopetal pathway that subserves morphine-induced analgesia. Stimulation of the PAG in the cat (Liebeskind et al, 1973; Sandkuhler, et al., 1988) and the rat (Bennett and Mayer, 1976) inhibited the responding of nociceptive neurons in the dorsal horn, as did stimulation of the NRM and the lateral reticular formation (Sandkuhler, et al., 1988). SPA was reduced by depletion of 5-HT with parachloralphenyalanine (p-CPA) and this reduction was reversed by the administration of the serotonin precursor 5-Hydroxytryptophan. Tetrabenzene, a compound that depletes all monoamines, almost totally abolished SPA, whereas increasing catecholamine levels with the catecholamine precursor L-DOPA potentiated SPA (Akil and Liebeskind, 1975). Electrical stimulation of the NRM and the NRPG caused the release of 5-HT and NE respectively into spinal cord

superfusates in the rat. (Hammond, Tyce and Yaksh, 1985). Analgesia evoked by stimulation of either the NRM or the NRPG has been shown to be attenuated by prior intrathecal administration of either methysergide, phenoxybenzamine, phentolamine or yohimbine (Satoh, et al., 1980; Hammond and Yaksh, 1984; Aimone, et al., 1987). This does not rule out the possibility that electrical stimulation of the PAG may also activate any number of other pain inhibition pathways.

It is likely that glutamate-produced analgesia mediated by the PAG is also modulated by the same bulbospinal system that mediates electrical SPA and morphine-induced analgesia. Glutamate administration to the PAG and the NRM but not the lateral reticular formation inhibited noxious heat evoked responses of dorsal horn nociceptive neurons (Sandkuhler et al., 1988). Furthermore, analgesia against a thermal noxious stimulus (Jensen and Yaksh, 1984) and a mechanical noxious stimulus (Satoh et al., 1983) produced by glutamate administration to both the PAG and the RVM was reversed by intrathecal administration of either phentolamine or methysergide. Analgesia produced by glutamate administration to the NRPG was reversed by intrathecal administration of the α-NE antagonists phenoxybenzamine or phentolamine but not methysergide or naloxone, while analgesia produced by glutamate administration to the NRM was antagonized by methysergide but not NE antagonists or naloxone (Satoh et al., 1983). A comparison of the analgesic effects of morphine and glutamate administration to the RVM showed that the analgesia induced by morphine, but not by glutamate, displayed a plateau (Jensen and Yaksh, 1989). These findings suggest that these medullary nuclei are involved in the mediation of both glutamate-produced and morphine-induced analgesia. However, they may have slightly different roles in the production of each type of analgesia. As was the case for electrical SPA, glutamate administration to the PAG may activate other antinociceptive pathways as well.

The results of Experiment 1 showed that the dorsal and ventral aspects of the PAG differentially support OA and GPA in developing animals. Morphine-induced analgesia was supported by ventral PAG sites in the neonatal animal, and by both dorsal and ventral sites in the more mature animal. Conversely, GPA was supported by dorsal sites in the neonate, and by both dorsal and ventral sites in the more mature animal. One interpretation of these data could be that the substrates within the dorsal and ventral PAG that mediate analgesia develop at different rates, but converge on the same monoaminergic bulbospinal system to produce analgesia in the more mature animal. The first goal of this study was to compare the degree to which morphine-induced and glutamate-produced analgesia mediated

by the dorsal or the ventral PAG could be attenuated by intrathecal administration of the serotonergic antagonist methysergide or the α-noradrenergic antagonist phentolamine.

There is evidence that noradrenergic spinal afferents originating in the RVM may modulate analgesia against mechanical noxious stimuli to a greater degree than thermal noxious stimuli. The converse may be true for serotonergic spinal afferents. Evidence for this came from the work of Kuraishi et al. in 1983. Depletion of spinal NE by intrathecal injections of the neurotoxin 6-hydroxydopamine (6-OHDA) attenuated the analgesic effects of morphine against mechanical, but not thermal stimuli. In contrast to this, depletion of spinal NE using 5,6-dihydroxytryptamine attenuated morphine-induced analgesia in response to thermal, but not mechanical noxious stimuli. Intrathecal administration of serotonin produced more robust analgesia against thermal noxious stimuli, whereas intrathecal administration of NE produced more profound analgesia against mechanical stimuli in adult rats (Kuraishi et al.,1985). Experiments performed in developing rats have supported these findings. Intraspinal 5,7-dihydroxytryptamine attenuated the analgesic effects of systemic morphine against a thermal stimulus to a greater degree than analgesia against a mechanical stimulus (Giordano and Barr, 1988). Intrathecal administration of norepinephrine or the α_2–noradrenergic agonist clonidine resulted in more pronounced analgesia against a mechanical than a thermal stimulus (Hughes and Barr, 1988). The results of Experiment 1 showed that glutamate administered to the PAG produced analgesia against a mechanical noxious stimulus earlier in development than against a thermal noxious stimulus, while the opposite effect was found when morphine was administered to the PAG. It is possible that when these compounds are administered to the PAG of very young animals, serotonergic and noradrenergic medullary nuclei are differentially activated, resulting in the more potent attenuation of either thermal or mechanical nociception. The second goal of this experiment was to assess the degree to which intrathecal methysergide or phentolamine would attenuate the analgesia produced by glutamate and morphine administration to the PAG against thermal and mechanical stimuli. This study was done using the 14 day old rat pup because previous experiments have indicated that this is the earliest age at which both glutamate-produced and morphine-produced analgesia supported by both dorsal and ventral PAG sites were apparent in the forepaw, hindpaw and tail of the developing rat based on the results of experiment 1.

Methods

Subjects

Fourteen day old pups were used as subjects in this experiment.

PAG cannula implants

Three 14 day old littermates were placed in an incubator maintained at 33° C until they were individually removed for surgery. The coordinates for the cannula implants were those described in Table 1 for dorsal and ventral PAG sites in 14 day old pups.

Intrathecal catheter implants

Methods for constructing and implanting intrathecal cannulae in developing rat pups have been described in detail elsewhere (Hughes and Barr, 1988). Briefly, catheters consisted of a flushed 1 cm length of Spectra/Por HF membrane (dialysis tubing with a 5000 MWCO) inserted halfway at one end into a 4 cm length of Dow Corning silastic medical grade tubing (.012 in ID, .025 in OD). The dialysis tubing was fastened to the silastic with a drop of cyanoacrylate cement applied at the interface between the two tubings. The entire catheter held approximately 3 µl of fluid. Prior to implantation, catheters were filled with saline to provide a measure of firmness to the tubing and to check for possible leaks. The dialysis end of the catheter were dipped in blue methylene dye (Sigma Chemical Co., St. Louis, Mo.) to enhance visibility prior to implant.

Following implantation of intraPAG catheters, and while pups were still under anesthesia, intrathecal catheters were implanted. A laminectomy at T-8 to T-11 was performed with care not to puncture the underlying dura. The dorsal surface of three vertebrae was removed in order to expose approximately 0.5 cm of spinal cord. The spinal dura was punctured by moving a hypodermic needle caudal in a plane parallel with the spinal axis until the tip pierced the dura. Approximately 0.25 cm of the needle was inserted and then removed. The dialysis tubing was inserted into the subdural space above the cord in a caudal direction. The silastic portion of the catheter was anchored by one drop of cyanoacrylate to the first intact vertebra rostral to the laminectomized area. The incision was closed with cyanoacrylate cement, allowing the the free end of the catheter to remain outside of the animal to permit microinjection of drug directly to the surface of the spinal

cord. After surgery, pups were returned to the incubator and housed separately to prevent dislodgement of cannulae through contact with siblings.

Intrathecal injections

Twenty hours after surgery pups were removed from the incubator. A 10 µl syringe was filled with 7 µl of drug solution and inserted into the exposed end of the catheter. Three µl of drug solution was injected into the catheter to fill the dead space, immediately followed by a steady injection of 4 µl of solution at a rate of 1 µl /15 seconds. The syringe was then removed. A drop of cyanoacrylate cement sealed the exposed end of the silastic to prevent the solution remaining in the catheter from seeping into the cord.

Analgesia Testing Procedures

Testing was done both prior to, and following, administration of drugs. The withdrawal latency of the forepaw, hindpaw and tail of each animal from noxious thermal and mechanical stimuli was measured using the methods described in Experiment 1. Thermal and mechanical tests were performed on limbs on the side of each animal that was contralateral to the side of the intrathecal cannula implant. This was to compensate for the damage to the dorsal horn on one side of the spinal cord caused by the intrathecal implant that might confound the assessment of analgesia in ipsalateral limbs.

Drug administration

Morphine Treatment -Morphine was administered to ventral PAG sites only in this experiment as previous studies have shown that morphine does not induce full analgesia in the 14 day old rat pup when given to the dorsal PAG. Pups assigned to the morphine treatment were divided into two groups. Each group consisted of three littermates from six litters. One group was given intraPAG morphine followed by intrathecal phentolamine, and the other group was given intraPAG morphine followed by intrathecal methysergide. Following baseline testing, pups were given and intraPAG injection of 0.6 μg of morphine in 0.5 μl of solution. IntraPAG injections were immediately followed by the intrathecal injection. Each pup in a litter assigned to the methysergide condition was given one of the following treatments in 4 μl of solution: saline vehicle, 15 μg of methysergide or 45 μg of methysergide. Each pup in the litters assigned to the phentolamine condition was given one of the following substances in 4 μl of solution: saline vehicle, 15 μg of phentolamine or 30 μg of phentolamine. Pups were then tested for their responses to the noxious stimuli as previously described. These doses were determined by dose response curves obtained in pilot studies.

Glutamate Treatment - Glutamate was administered to both dorsal and ventral aspects of the PAG in this experiment. This is because previous experiments showed that both dorsal and ventral PAG regions support glutamate-produced analgesia in the 14 day old rat pup. Six groups of three littermates received dorsal PAG implants and six groups of three littermates received ventral PAG implants in both the phentolamine and methysergide conditions. Following baseline testing, pups were given an intraPAG injection of 180 mM of glutamate in 0.5 μl of solution. IntraPAG injections were immediately followed by intrathecal injections of either phentolamine or methysergide administered in the same doses and in the same manner as in the morphine treatment.

Experimental Design

Experimental units were made up of three 14 day old littermates (N=3). Six units were assigned to each of the following conditions: glutamate administered to the dorsal PAG and intrathecal (i.t.) phentolamine (N=18), glutamate administered to the dorsal PAG and i.t. methysergide (N=18), glutamate administered to the ventral PAG and i.t. phentolamine (N=18), glutamate administered to the ventral PAG and i.t. methysergide (N=18), morphine administered to the ventral PAG and i.t. phentolamine (N=18) or morphine administered to the ventral PAG and i.t. methysergide (N=18). Each pup in a unit assigned to the intraPAG glutamate (N=3) or morphine (N=3) and methysergide condition was given either 180 mM of glutamate or 6 μg of morphine to the PAG. One pup per unit was given i.t. saline and served as a control, one pup was given a low dose of i.t. methysergide (15 μg), and the third pup was given a high dose of i.t. methysergide (45 μg). Each pup in a unit assigned to the intraPAG glutamate (N=3) or morphine (N=3) and phentolamine condition was given either 180 mM of glutamate or 6 μg of morphine to the PAG. One pup per unit was given i.t. saline as a control, one pup was given a low dose of i.t. phentolamine (15 μg), and the third pup was given a high dose of i.t. phentolamine (30 μg).

Histology

Pups were perfused with 0.9% saline for approximately 1 minute, immediately followed by perfusion with approximately 15 ml of 10% formaldehyde administered over a period of 10 minutes. Animals were decapitated and brains removed and stored in 70% alcohol until they were removed for sectioning. Brains were dehydrated, embedded in paraffin, blocked, and 10 μm sections were taken in the area of the PAG. Slides were stained with Cresyl Violet, coverslipped, and examined under a light microscope to verify PAG implant sites

Statistics

Data were analyzed using two analyses of variance. The first compared the effects of intrathecal methysergide and phentolamine on analgesia produced by morphine and glutamate administered to the ventral PAG. The factors analyzed were PAG drug (morphine or glutamate), intrathecal drug (methysergide or phentolamine), dose of intrathecal drug (vehicle, low or high), stimulus type (thermal or mechanical) and body part (forepaw, hindpaw or tail). The second analysis compared the effects of intrathecal methysergide and phentolamine on the analgesia produced by glutamate administered to the dorsal and ventral PAG. The factors for this analysis were: site within the PAG (dorsal or ventral), intrathecal drug (methysergide or phentolamine), dose of intrathecal drug (vehicle, low or high), stimulus type (thermal or mechanical) and body part (forepaw, hindpaw or tail). A post hoc Tukey test was performed following each analysis to clarify the nature of significant interactions. The results of the first analysis are summarized in Table 6 and the results of the second analysis are summarized in Table 7.

Table 6. This table shows the results of the analyses of variance for the data obtained when morphine and glutamate were administered to the ventral PAG in Experiment 3. Abbreviations are as follows: I is intrathecal drug (Methysergide or Phentolamine), P is PAG drug (Morphine or Glutamate), S is stimulus type (Thermal or Mechanical), D is dose of intrathecal drug (Vehicle, Low, or High) and B is Body Part (Forepaw, Hindpaw or Tail).

	Sum of Squares	Degrees of Freedom	Mean Square	F	Tail Probability
I	0.31579	1	0.31579	0.12	0.7356
P	0.02613	1	0.02613	0.01	0.9225
IP	2.71701	1	2.71701	1.01	0.3272
S	6.99722	1	6.99722	5.26	0.328
SI	107.88003	1	107.88003	81.07	0.0000
SP	0.020803	1	0.020803	0.02	0.8861
SIP	0.72193	1	0.72193	0.54	0.4699
D	5074.18975	2	2537.09488	1281.52	0.0000
DI	0.81790	2	0.40895	0.21	0.8142
DP	7.94251	2	3.17925	2.01	0.1478
DIP	12.84909	2	6.42455	3.25	0.0494
SD	5.86085	2	2.93043	2.36	0.1074
SDI	278.58343	2	139.29171	112.18	0.0000
SDP	3.32534	2	1.66267	1.34	0.2736
SDIP	1.47862	2	0.73931	0.60	0.5562
B	11.09667	2	5.54833	7.14	0.0022
BI	0.74761	2	0.37381	0.48	0.6218
BP	0.45286	2	0.22643	0.29	0.7489
BIP	0.91233	2	0.45616	0.59	0.5608
SB	4.64216	2	2.32108	2.43	0.1014
SBI	0.03253	2	0.01626	0.02	0.9832
SBP	3.03750	2	1.51875	1.59	0.2172
SBIP	0.37020	2	0.18510	0.19	0.8249

DB	4.56292	4	1.14073	1.91	0.1173
DBI	5.45485	4	1.36371	2.28	0.0679
DBP	0.98122	4	0.24530	0.41	0.8009
DBIP	6.75828	4	1.68957	2.82	0.0302
SDB	2.13828	4	0.53457	0.66	0.5684
SDBI	5.07606	4	1.26902	1.56	0.2118
SDBP	1.26156	4	0.31539	0.39	0.7445
SDBIP	6.94161	4	1.73540	2.14	0.1112

Table 7. This table shows the results of the analyses of variance for the data obtained when glutamate was administered to the dorsal and ventral PAG in Experiment 3. Abbreviations are as follows: I is intrathecal drug (Methysergide or Phentolamine), P is PAG location (Dorsal or Ventral) S is stimulus type (Thermal or Mechanical), D is dose of intrathecal drug (Vehicle, Low, or High) and B is Body Part (Forepaw, Hindpaw or Tail).

	Sum of Squares	Degrees of Freedom	Mean Square	F	Tail Probability
I	5.86367	1	5.86367	1.36	0.2577
P	0.44403	1	0.44403	0.10	0.7518
IP	12.30525	1	12.30525	2.85	0.1070
S	0.00265	1	0.00265	0.00	0.9692
SI	42.92191	1	42.92191	24.81	0.0001
SP	7.62410	1	7.62410	4.41	0.0487
SIP	21.94657	1	21.94657	12.69	0.0020
D	4907.87888	2	24534.939	692.07	0.0000
DI	36.48217	2	18.24109	5.14	0.0103
DP	47.38983	2	23.69492	6.68	0.0031
DIP	14.25398	2	7.12699	2.01	0.1473
SD	0.50836	2	0.25193	0.21	0.8101
SDI	119.77709	2	59.88884	50.33	0.0000
SDP	9.36259	2	4.68130	3.93	0.0276
SDPI	48.82519	2	24.41260	20.52	0.0000
B	10.38913	2	5.19456	6.29	0.0042
BI	6.16658	2	3.08329	3.73	0.0327
BP	0.16212	2	0.08106	0.10	0.9068
BIP	3.89318	2	1.94659	2.36	0.1079
SB	5.64302	2	2.82151	2.99	0.0617
SBI	0.83001	2	0.41500	0.44	0.6473
SBP	0.43024	2	0.21512	0.23	0.7972
SBIP	0.26130	2	0.10815	0.11	0.8920

DB	4.14098	4	1.03524	1.04	0.3909
DBI	6.26550	4	1.56638	1.58	0.1886
DBP	1.96596	4	0.49149	0.49	0.7396
DBIP	3.91652	4	0.97913	0.99	0.4203
SDB	3.70571	4	0.92643	1.34	0.2624
SDBI	2.28080	4	0.57020	0.82	0.5133
SDBP	0.51774	4	0.12944	0.19	0.9444
SDBIP	4.75250	4	1.18813	1.72	0.1541

Results

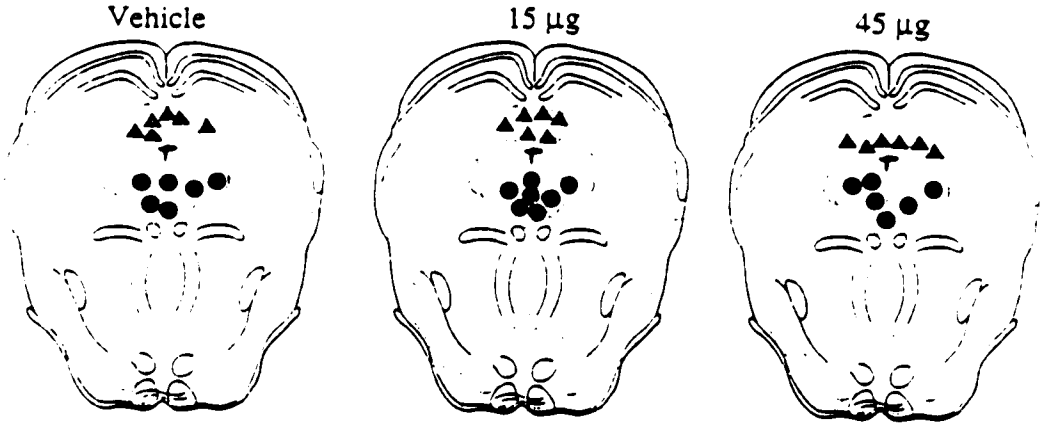
Implant sites

Glutamate was administered to dorsal PAG sites in a total of 18 pups and to ventral PAG sites in a total of 18 pups for each of the two intrathecal drug treatments. Figure 7 shows the sites to which glutamate was administered in this experiment. Morphine was administered to a total of 18 ventral PAG sites for each of the two intrathecal drug treatments. The sites to which morphine was administered can be seen in Figure 8.

Figure 7. Sites of glutamate administration to the periaqueductal grey area prior to intrathecal administration of the serotonergic antagonist methysergide or the α–noradrenergic antagonist phentolamine.

Glutamate

Methysergide



Phentolamine

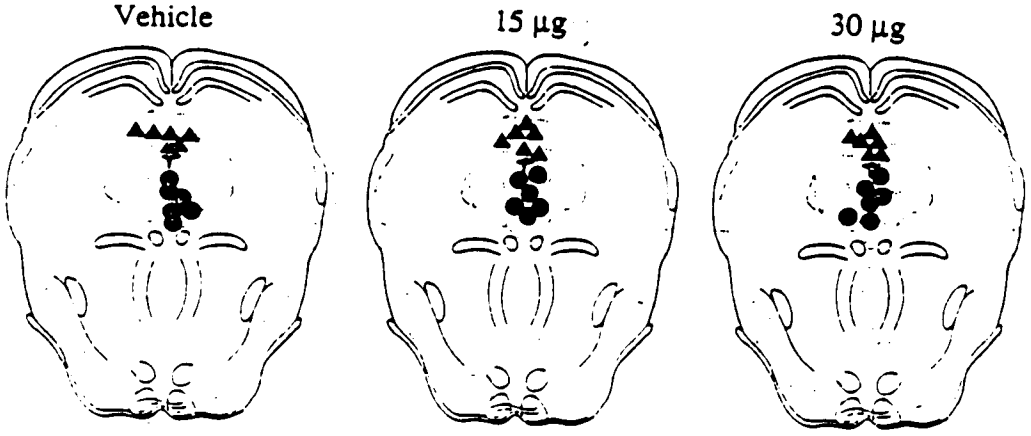
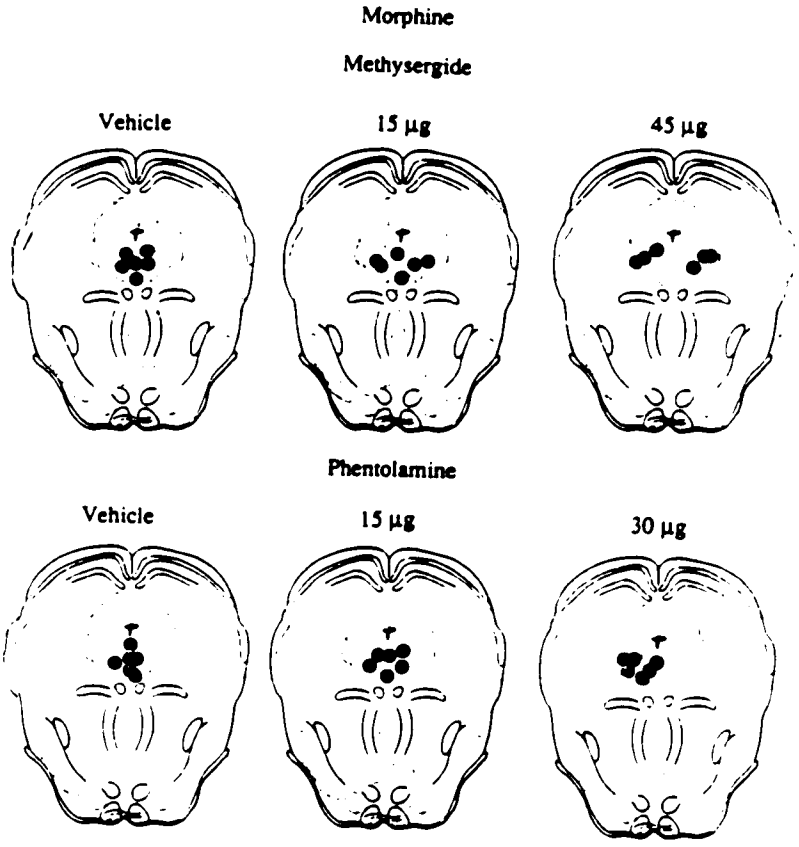


Figure 8. Sites of morphine administration to the periaqueductal grey area prior to intrathecal administration of the serotonergic antagonist methysergide or the α–noradrenergic antagonist phentolamine.

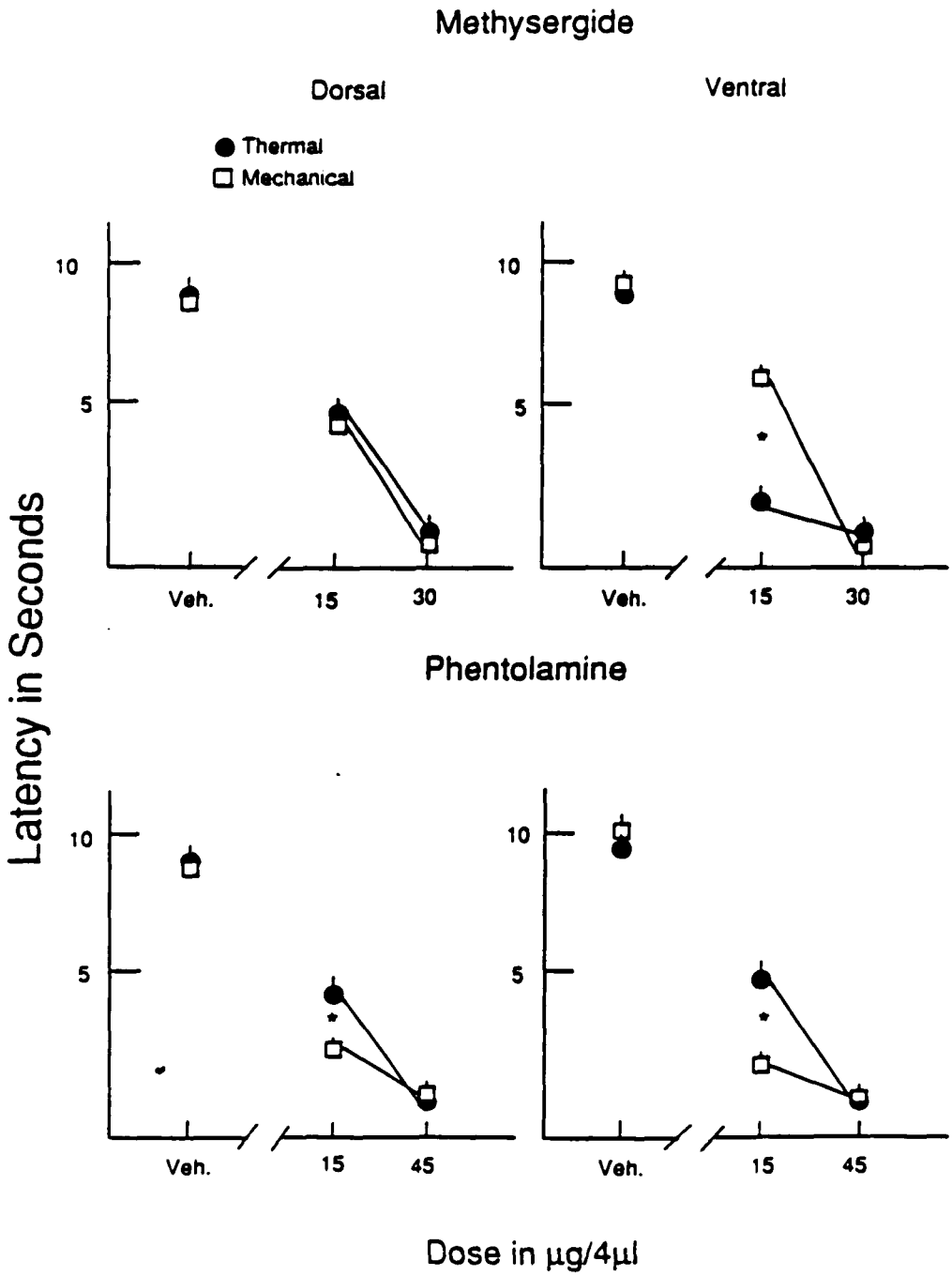

Morphine
Methysergide
Vehicle
15 µg
45 µg
Phentolamine
Vehicle
15 µg
30 µg

Reversal of glutamate-produced analgesia

When glutamate was administered to dorsal PAG sites, the analgesia produced against the thermal stimulus was reversed in a dose dependent manner by intrathecal phentolamine. The low dose of phentolamine attenuated glutamate-produced analgesia against the mechanical stimulus to a signifcantly greater degree by intrathecal administration of than was the analgesia produced against the thermal stimulus. The high dose of intrathecal phentolamine reduced the level of analgesia produced by glutamate against both types of stimuli to baseline levels. When glutamate was given to the dorsal PAG, intrathecal methysergide anatagonized the analgesia produced against both types of stimuli in a does dependent manner. These results can be found in Figure 9.

When glutamate was given to the ventral PAG, analgesia produced against the thermal stimulus was reduced in a dose dependent manner by intrathecal phentolamine. Analgesia produced against the mechanical stimulus was attenuated to a significantly greater degree than the analgesia against the thermal stimulus by the low dose of phentolamine. The high dose of phentolamine attenuated glutamate-produced analgesia against both stimulus types to baseline levels. When glutamate was given to the ventral PAG, the analgesia produced against the thermal stimulus was reversed to a significantly greater than was the analgesia produced against the mechanical stimulus by the low dose of intrathecal methysergide. The high dose of methysergide antagonized the effects of glutamate to baseline withdrawal levels. These results are displayed in Figure 9.

Figure 9. The effects of intrathecal phentolamine on the analgesia produced by glutamate administration to the dorsal and ventral PAG of 14 day old rats. Withdrawal latencies against thermal and mechanical stimuli are shown.


Methysergide
Dorsal
Ventral
Thermal
Mechanical
10
5
Veh.
15
30
Phentolamine
45
Latency in Seconds
Dose in µg/4µl

Reversal of morphine-induced analgesia

Morphine was exclusively administered to the ventral aspect of the PAG in this experiment. The low dose of phentolamine attenuated morphine-induced analgesia against the mechanical stimulus to a significantly greater degree than analgesia against the thermal stimulus.The high dose of phentolamine administered intrathecally was sufficient to completely abolish morphine-induced analgesia against both stimulus types. The low dose of methysergide administered intrathecally attenuated morphine-induced analgesia against the thermal noxious stimulus to a significantly greater degree than morphine-induced analgesia against the mechanical stimulus. The high dose of methysergide reversed morphine-induced analgesia against both types of stimuli to baseline levels. These results are graphically represented in Figure 10. The results of this experiment are summarized in Table 8.

Figure 10. The effects of intrathecal phentolamine and methysergide on morphine-induced analgesia supported by the ventral PAG of 14 day old rats. Withdrawal latencies from thermal and mechanical noxious stimuli are shown.

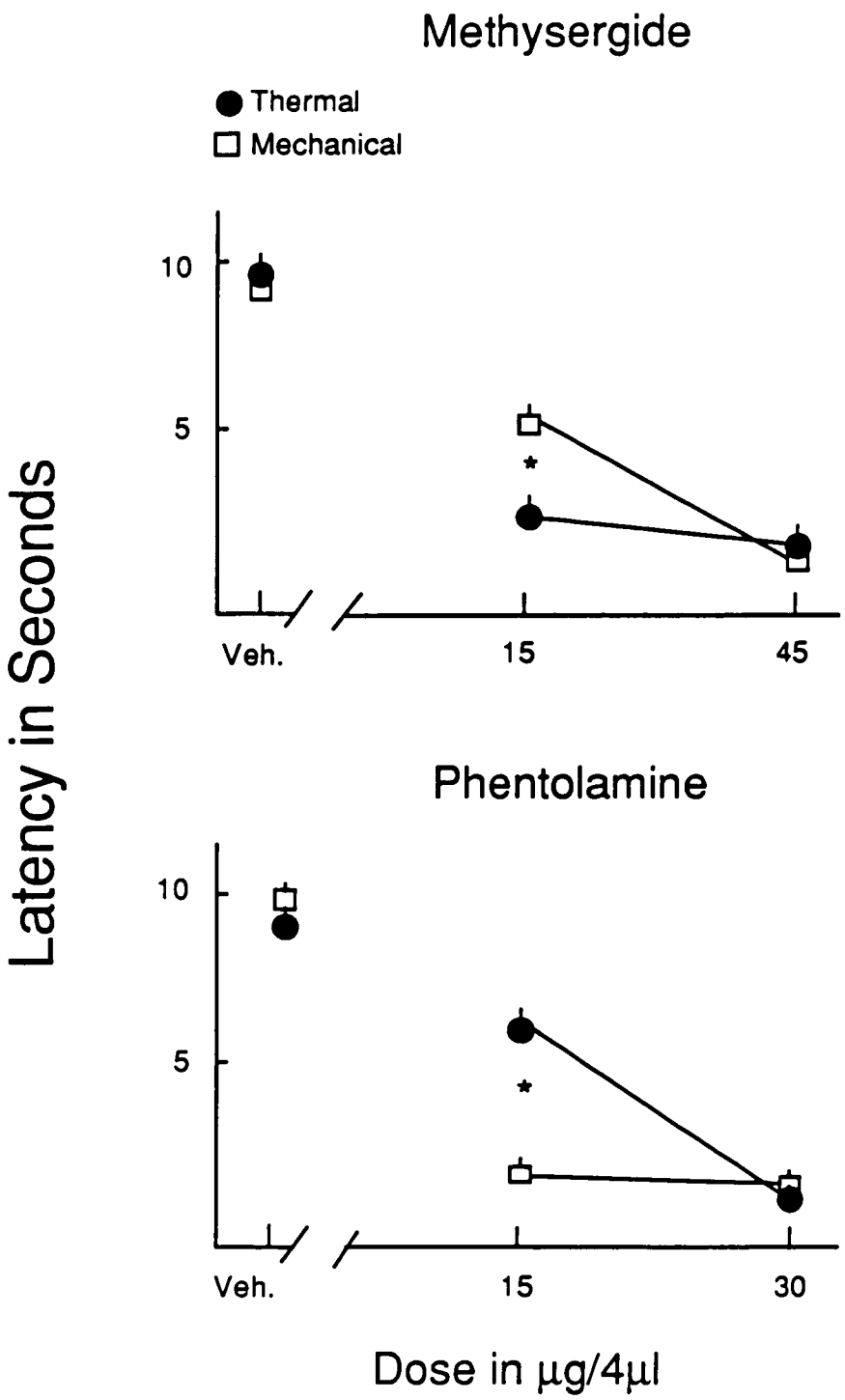

Methysergide
Thermal
Mechanical
10
5
Veh.
15
45
Phentolamine
10
5
Veh.
15
30
Latency in Seconds
Dose in µg/4µl

Table 8. This table summarizes the results of Experiment 3. + indicates a significant attenuation of analgesia as compared with levels measured for controls. ++ indicates significant attenuation of analgesia as compared with levels measured for the other stimulus type.

Glutamate
Dorsal

Methysergide 15 µg		Phentolamine 15 µg	
Thermal	Mechanical	Thermal	Mechanical
+	+	+	++

Glutamate
Ventral

Methysergide 15 µg		Phentolamine 15 µg	
Thermal	Mechanical	Thermal	Mechanical
++	+	+	++

Morphine
Ventral

Methysergide 15 µg		Phentolamine 15 µg	
Thermal	Mechanical	Thermal	Mechanical
++	+	+	++

Discussion

This study compared the effects of intrathecal serotonergic and α–noradrenergic antagonists on glutamate-produced and morphine-induced analgesia mediated by sites within the dorsal and ventral PAG. The results showed that glutamate-produced and morphine-induced analgesia supported by the ventral PAG can be attenuated by intrathecal administration of the serotonergic antagonist methysergide or by the α-noradrenergic antagonist phentolamine. Regardless of whether morphine or glutamate had been administered to the ventral PAG, the low dose of intrathecal methysergide attenuated analgesia produced by that compound against the thermal noxious stimulus to a significantly greater degree than against the mechanical noxious stimulus. However, when glutamate was given to the dorsal PAG, intrathecal methysergide attenuated the antinociceptive effects of both compounds to the same degree against both types of stimuli. In contrast to this, the low dose of phentolamine attenuated the analgesic effects of both intraPAG compounds against the mechanical noxious stimulus to a greater degree than the thermal stimulus. The stimulus specific effects of intrathecal phentolamine were seen when glutamate was administered to the dorsal PAG and when morphine or glutamate were given to the ventral PAG.

Several conclusions may be drawn from these results. The first is that both morphine-induced and glutamate-produced analgesia supported by the PAG are mediated by common bulbospinal monamine systems. This was evident because intrathecal monoamine antagonists were shown to reverse the antinociceptive effects of both compounds administered to the PAG. These findings are in keeping with the findings of other laboratories that have shown that morphine-induced and glutamate-produced analgesia mediated by supraspinal sites can be antagonized by monoaminergic antagonists administered to the spinal cord (Jensen and Yaksh, 1984,1986; Satoh, 1983).

The results of this study also support the concept of the functional subdivision of the PAG into a dorsal and a ventral region. In previous experiments we showed that the OA and GPA supported by the dorsal and ventral PAG developed at different rates . Other laboratories also found differences in the analgesia supported by dorsal and ventral PAG regions in the adult animal (Akil et al., 1976; Lewis and Gebhart, 1977; Cannon et al., 1982; Fardin, et al., 1984; Morgan and Liebeskind, 1987; Prieto et al., 1983). In this experiment, differences between the dorsal and ventral PAG were seen most clearly when the serotonergic antagonist methysergide was used to antagonize glutamate-produced

analgesia from dorsal and ventral PAG sites. The low dose of methysergide reversed glutamate-produced analgesia from the ventral PAG in a stimulus specific manner. The analgesia produced against the thermal stimulus was antagonized to a significantly greater degree than was the analgesia against the mechanical stimulus. In contrast to this, when glutamate was administered to the dorsal PAG the analgesia produced against both types of noxious stimuli were reversed to the same degree and in a dose dependent manner by the low dose of intrathecal methysergide. Thus, the analgesia produced by glutamate administration to the dorsal and ventral PAG appear to be differentially modulated by spinal serotonergic systems.

The results of this experiment support the hypothesis that the analgesia produced against thermal and mechanical stimuli by opiate administration to, or stimulation of, the PAG or nuclei in the RVM are differentially modulated by spinal serotonergic and noradrenergic systems (Kuraishi et al., 1983,1985). The low dose of phentolamine produced a more potent antagonism of the analgesia produced by both morphine and glutamate against the mechanical noxious stimulus, while the low dose of methysergide more effectively antagonized analgesia against the thermal noxious stimulus when morphine or glutamate were given to the ventral PAG. These findings imply that spinal norepinephrine usually modulates analgesia against mechanical pain more effectively, while spinal serotonin modulates analgesia against thermal pain more effectively. The hypothesis that treatments that stimulate the release of norepinephrine into the spinal cord produce more robust analgesia against mechanical noxious stimuli, whereas treatments that cause the release of serotonin into the spinal cord induce more potent analgesia against thermal noxious stimuli has been tested previously. The administration of morphine to the noradrenergic NRGC produced potent dose dependent analgesia against a mechanical noxious stimulus that was thought to be modulated by the release of norepinephrine into the spinal cord (Kuraishi et al., 1979). In addition, glutamate administration to the PAG or to noradrenergic nuclei within the rostral ventral medulla produced robust dose dependent analgesia against a mechanical noxious stimulus. This analgesia was also thought to be modulated by spinal norepinephrine (Satoh et al., 1983). The same treatments also produced analgesia against a thermal noxious stimulus as did stimulation of the NRM by glutamate or morphine (Jensen and Yaksh, 1984, 1986). Thus, it is likely that the more potent analgesic effects induced by spinal norepinephrine against a mechanical stimulus or by spinal serotonin against a thermal stimulus can best be seen when analgesia against both types of stimuli are simultaneously compared.

Several investigators (Yaksh, 1979; Jensen and Yaksh, 1984,1986; Sandkuhler and Zimmermann, 1988) have found that the effects of stimulating the PAG electrically, or with glutamate or morphine were only partially reversed by the intrathecal administration of either phentolamine or methysergide. The analgesic effects of electrically stimulating the PAG , as well as the inhibitory effect of this treatment on cells in the spinal cord dorsal horn, were fully antagonized only when a combination of methysergide and phentolamine were administered to the spinal cord. In the present experiment, full attenuation of the analgesic effects of intraPAG morphine or glutamate was obtained by the intrathecal administration of the high dose of either phentolamine or methysergide alone. These findings are paradoxical as spinal NE and 5-HT are thought to work in tandem to produce analgesia. Therefore, blocking one neurotransmitter system or the other should theoretically not be sufficient to totally abolish analgesia. One possible cause for these findings is that while phentolamine and methysergide may have higher affinities for the noradrenersic and serotonergic receptor types respectively, they may also be weak antagonists at both receptor types. Therefore, when a high dose of either of these compounds is administered, both noradrenergic and serotonergic receptors might be blocked to a degree sufficient to totally abolish analgesia from the PAG. However, previous experimenters (Yaksh, 1979; Jensen and Yaksh, 1984,1986; Sandkuhler and Zimmermann, 1988) have given intrathecal doses of methysergide and phentolamine that were equally as high as the doses used in the present study, yet they were not able to totally abolish analgesia from the PAG. This implies that the spinal receptor populations for serotonin and norepinephrine in developing animals are structurally or at very least functionally different from those in the adult. Two hypotheses can be proposed to explain these findings. One hypothesis is that receptor sites in the neonatal pups are structurally distinct from adult receptor populations. There may be fewer differences in the protein congfigurations of the two receptor types in developing animals, thereby allowing both types of receptor types to more efficaciously bind both types of compounds. An alternative hypothesis is that there are not differences in the qualitative nature of these receptor types, but rather in the relative quantities of these receptor types in developing versus adult animals. If there were fewer receptors for both serotonin and norepinephrine in the spinal cords of developing animals, then there would be an increased probability that a compound with a strong affinity for one type of receptor and a weak affinity for the other type of receptor might bind to and antagonize both receptor types if a high enough dose were given. Either of these hypotheses would explain why the analgesia produced by PAG manipulations is not fully blocked by the intrathecal administration of either of these monoamine anatagonists alone in the adult animal and a combination of both methysergide

and phentolamine is required to reverse this analgesia. These hypotheses should be investigated in the future using autoradiography.

In this experiment, the low dose of methysergide differentially attenuated the glutamate-produced analgesia supported by dorsal and ventral PAG sites. The reasons for this difference are not known, although hypotheses can be proposed. Projections from the dorsal and ventral PAG were examined by Beitz in 1985. He found that cells originating in the ventral PAG projected more profusely to the NRM than did cells in the dorsal PAG. Projections from the dorsal PAG were found to be more evenly distributed between the NRM, the NRGC and the NRPG. Thus, it is possible that when cells in the ventral PAG were stimulated by glutamate, serotonergic cells within the NRM that project to the spinal cord might have been activated to a greater degree than cells within the reticular nuclei. This would produce a larger efflux of serotonin than of norepineprine into the spinal cord. The presence of relatively high high levels of serotonin within the spinal cord in response to ventral PAG stimulation could cause the effects of the serotonergic antagonist methysergide to be more pronounced. Glutamate given to the dorsal PAG might cause the release of less serotonin into the spinal cord than does glutamate administration to the ventral PAG. Under these conditions, the stimulus specific antagonism of analgesia produced by intrathecal methysergide might be less pronounced. Phentolamine antagonized the effects of glutamate given to both the dorsal and ventral PAG sites and the effects of morphine given to ventral PAG sites in the same manner and to the same degree. This might be due to the fact that dorsal and ventral PAG stimulation induce similar spinal norepinephrine levels.

Jensen and Yaksh (1984, 1986) proposed that the intrathecal administration of monoaminergic antagonists attenuates the analgesia produced by glutamate or morphine administration to the PAG against spinally, but not supraspinally mediated responses. Specifically, they found that intrathecal methysergide or phentolamine successfully reversed analgesia produced by intraPAG morphine or glutamate against the spinally organized tail flick response, but that intrathecal administration of these compounds had no effect on the analgesia produced against the supraspinally organized hot plate response. In contrast to this, others have found that intrathecal administration of these monoamine antagonists effectively attenuated both glutamate-produced and morphine-induced analgesia against a supraspinally organized response to tail pinch. In this test analgesia was measured by an increase in the latency for biting forceps that were pinching the tail (Kuraishi et al., 1979; Satoh et al., 1983). An explanation proposed to explain these

differering results was the differential use of thermal and mechanical algesic tests in these studies (Jensen and Yaksh, 1984). The present study measured both spinally and supraspinally organized withdrawal responses from thermal and mechanical noxious stimuli. We have determined that the withdrawal of the tail from the thermal or mechanical stimuli used in these experiments is a spinally organized response. When the spinal cord was cooled at the lumbar-thoracic level, the withdrawal of the tail from noxious thermal and mechanical stimuli persisted. However, the withdrawal of the hindpaw from these noxious stimuli was abolished (Paredes and Barr, unpublished observations) after spinal cord cooling. The withdrawal of the forepaw from these noxious stimuli could only be blocked by cooling the spinal cord at levels that also interfered with respiration, so it is not known whether this response is organized at spinal or supraspinal levels. Thus, our testing paradigm allowed us to observe the effects of intrathecal methysergide and phentolamine on analgesia produced against both thermal and mechanical noxious stimuli utilizing both spinally and supraspinally mediated withdrawal responses. The results showed that intrathecal methysergide and phentolamine effectively attenuate morphine-induced and glutamate-produced analgesia against both thermal and mechanical stimuli as measured by both spinally and supraspinally organized responses. Therefore, the differences in the results obtained by Jensen and Yaksh (1984) and those obtained by Kuraishi et al.(1979) and Satoh et al.(1983) may not be solely attributable to the type of noxious stimulus employed. Our results support the findings of Kuraishi et al. and Satoh et al. Intrathecal monoamine antagonists attenuated analgesia to stimuli measured using supraspinally organized withdrawal responses. The mechanisms by which this occurs have yet to be elucidated.

In conclusion, the results of the present study show that glutamate and morphine administered to the PAG both produce analgesia by activating monoaminergic projections that terminate in the spinal cord. The analgesia produced by either drug administered to the PAG can be reversed by the intrathecal administration of the serotonergic antagonist methysergide or the noradrenergic α antagonist phentloamine. Intrathecal phentolamine antagonized analgesia against the mechanical stimulus more effectively while intrathecal methysergide more effectively reversed analgesia against the thermal noxious stimulus. These findings indicate that spinal norepinephrine may modulate antinociception against mechanical noxious stimuli to a greater degree than against athermal painful stimulus, while the opposite may be true for spinal serotonin.

General Discussion

Pain and analgesia assessment in animals

The measurement of pain and analgesia in animals is a very complex task. It is difficult to know whether an increase in withdrawal latency is occuring because the animal is analgesic or due to some other extraneous reason. In this thesis analgesia was very narrowly defined as a significant increase in withdrawal latency following limb or tail exposure to noxious thermal or mechanical stimuli. Despite this restrictive definition, the issue of whether or not this increased latency is due to diminished pain perception persists. The increased withdrawal latencies seen in these studies are thought to represent analgesia for several reasons. The same stimulus intensities were used in these experiments as in those performed by Hughes and Barr (1988). They have shown that these intensities were the lowest needed to produce reliable withdrawal responses in young rats. Stimuli of lower intensities did not produce withdrawal responses indicating that these stimuli were not noxious to the animals tested. Perception of stimuli as noxious and the desire to withdraw abruptly from them is the closest approximation of pain in animals that we can have. Control groups that were given saline vehicle rather than morphine or glutamate responded no differently to these noxious stimuli than did untreated animals. However, when glutamate or morphine was given to the PAG, the animals did not withdraw their limbs or tails from the stimuli as rapidly. Therefore, the administration of these compounds to the PAG changed the animals response to these stimuli, although the stimuli themselves did not change. In addition to this rather circumstantial evidence, the studies in this thesis showed differences in the withdrawal of limbs and tail from thermal and mechanical noxious stimuli under certain sets of circumstances. Specifically, intraPAG morphine increased withdrawal latencies from thermal stimuli at an earlier age than from mechanical stimuli while the converse was true for intraPAG glutamate. Moreover, methysergide and phentolamine differentially decreased withdrawal latencies from thermal and mechanical stimuli. These findings indicate that the animals differentially respond to thermal and mechanical noxious stimuli. Furthermore, the fact that certain treatments increase or decrease withdrawal latencies from one type of stimulus without effecting withdrawal from the other type strongly suggests that antinociception is being produced against a specific noxious quality of the stimulus itself and cannot be attributed to other things such as general motor deficits or anesthesia. For example, if 3 day old rat pups given intraPAG morphine are able to try to withdraw their forepaws from a mechanical stimulus with latencies of less than one second but do not withdraw their

forepaw from a thermal bath, we can be quite sure that this lack of response is not due to motor impairment or general anethesia. These findings allow some amount of confidence when speaking of increased withdrawal latencies as analgesia.

Reexamination of the goals of this thesis

The goal of this thesis was to answer several questions regarding the development of OA and GPA mediated by the PAG. The primary question asked by this thesis was whether the PAG could be functionally divided into a dorsal and ventral region with regard to its ability to support opiate and non-opiate forms analgesia. This question was addressed in all of the experiments that were done. The results of Experiment 1 showed that morphine and glutamate produced different effects depending upon whether they were given to the dorsal or the ventral PAG in developing rats. The analgesia against thermal and mechanical stimuli produced by these compounds when focally administered to the dorsal or the ventral PAG developed at different rates. This indicates that a functional demarcation between sites within the dorsal and ventral PAG does exist in the developing animal and probably persists in mature animals as well. The results of Experiment 2 indicated that systemic naloxone effectively blocked both glutamate-produced and morphine-induced analgesia from the ventral PAG but had no effect on analgesia mediated by the dorsal PAG. This supports the hypothesis that analgesia subserved by the dorsal and ventral PAG are differentially mediated. One possible mechanism for the site specific effects of naloxone is that projections from the ventral PAG to nuclei in the RVM may be predominantly enkephalinergic while the projections from the dorsal PAG to the RVM may be predomintly excitatory. The third experiment tested the hypothesis that differences in the analgesia supported by dorsal and ventral PAG sites were due to differential projections from the PAG to nuclei in the rostral ventral medulla. The results showed that although there were slight differences in the ability of phentolamine and methysergide to reverse GPA from dorsal and ventral sights, analgesia from both dorsal and ventral PAG sights was dependent on spinal serotonin and norepinephrine. Therefore, differences in analgesia supported by dorsal and ventral PAG sights could not be attributed to differential projections to the RVM nuclei.

The differential effects of morphine and glutamate administered to the dorsal and ventral PAG evidenced early in development could occur for several reasons. One reason is that glutamate sensitive neurons and the morphine sensitive neurons within the PAG may have different receptive fields within the PAG. Another possibility is that these neurons may

synapse on distinct areas of the RVM in developing animals, and perhaps in adult animals as well. It is also possible that differences in the growth rate of bulbospinal projections arising from different nuclei witihin the RVM may account for some of these differences. Different subtypes of opiate and glutamate receptors located within the PAG might be responsible for the developmental differences seen in the production of analgesia as well. These receptor subtypes may emerge at different rates and be responsible for the differences between the production of analgesia by glutamate or morphine within the PAG during development. Another possibility is that the developmental differences found in this experiment reflect the formation of local circuits within the PAG.

In addition to addressing the dorsal-ventral question, this thesis also sought to determine whether glutamate and morphine act in the same manner within the PAG to produce analgesia. It is possible that receptors for glutamate and morphine coexist on the same neurons within the PAG and that stimulation of these cells produces analgesia by way of bulbospinal pathways. The results of Experiment 1 argue against this hypothesis. In Experiment 1 it was shown that glutamate and morphine do not produce analgesia when given to the same sites within the PAG in the developing animal. This finding strongly suggests that these compounds act on different cells within the PAG. The results of Experiment 1 showed that glutamate and morphine produce analgesia against different types of analgesia in the developing animal. If glutamate and morphine were acting on the same substrates within the PAG, they would be expected to produce analgesia against the same types of stimuli at all times. Taken together, the results of Experiment 1 strongly suggest that morphine and glutamate do not act in the same manner within the PAG to produce analgesia. Therefore, alternate mechanisms of action for these compounds have been proposed. Morphine administered to the PAG is thought to inhibit tonic inhibition on neurons in the RVM normally imposed by GABAergic neurons projecting from the PAG to cells in the RVM. Conversely, glutamate administered to the PAG probably directly excites an excitatory pathway descending from cells in the PAG to cells in the RVM. Thus, the predominant role of morphine in the PAG with regard to descending antinociceptive nociceptive pathways is one of disinhibition, whereas the role of glutamate with regard to these pathways is direct excitation.

A third question proposed in this thesis was whether or not GPA and OA mediated by both the dorsal and the ventral PAG were subserved by the same descending bulbospinal systems. The third experiment addressed this question by attempting to reverse GPA from both dorsal and ventral PAG sites and OA from ventral PAG sites with intrathecally

administered methysergide or phentolamine in 14 dat old rats. If GPA and OA mediated by the PAG are subserved by the same bulbospinal systems, intrathecal administration of these serotonergic and noradrenergic antagonists should reverse the analgesia produced by both compounds to the same degree and in the same manner. If GPA and OA are not modulated by the same spinopetal systems, the effects of these intrathecal antagonists on the analgesia they produce should differ. The results showed that when morphine or glutamate were given to the ventral PAG the low dose of methysergide attenuated analgesia against the thermal stimulus to a significantly greater degree. Phentolamine attenuated analgesia produced by both compounds against the mechanical stimulus to a significantly greater degree. The finding that intrathecal phentolamine and methysergide have the same effects on GPA and OA indicates that both intraPAG compounds produce analgesia by way of the same spinopetal pathways in the more mature animal despite their differential development.

Another goal of this thesis was to determine whether the monoamine neurotransmitters released into the spinal cord following intraPAG glutamate or morphine administration differentially mediated analgesia against thermal and mechanical noxious stimuli. In Experiment 1 it was shown that intraPAG glutamate produced analgesia only against the mechanical stimulus at younger ages, whereas morphine specifically produced analgesia against thermal stimuli in young animals. One parsimonious explanation for these findings is that the excitatory pathway activated by glutamate projects to noradrenergic reticular nuclei in the neonatal animal, and projections of these excitatory neurons to serotonergic cells in the NRM do not develop until later. It is also possible that the inhibitory pathway stimulated by morphine projects predominantly to serotonergic cells in the NRM early in development and projections to noradrenergic reticular nuclei are not fully formed until later ages. If this hypothesis is accurate, we would expect to see GPA against the mechanical stimulus and OA against the thermal stimulus early in development. Both compounds would be expected to produce analgesia against both types of stimuli in mature animals. Thus, the results of Experiment 1 support the concept that serotonergic and noradrenergic projections from nuclei in the RVM to the spinal cord might differentially modulate analgesia against thermal and mechanical stimuli without directly testing this hypothesis.

Experiment 3 was done in order to gain more information regarding the relative roles of spinal 5-HT and NE in GPA and OA mediated by the PAG. In Experiment 3, the serotonergic antagonist methysergide or the α-noradrenergic antagonist phentolamine were given intrathecally in conjunction with intraPAG glutamate or morphine. If spinal serotonin more potently attenuates the perception of noxious thermal stimuli and spinal

norepinephrine more potently attenuates nociception of mechanical stimuli, we would expect intrathecal methysergide and phentolamine to more effectively antagonize OA and GPA against thermal and mechanical stimuli respectively. This was indeed found to be the case. With the exception of when glutamate was given to the dorsal PAG, the low dose of methysergide consistantly attenuated OA and GPA against the thermal stimulus to a greater degree than the mechanical stimulus. The low dose of phentolamine always attenuated OA and GPA against the mechanical stimulus to a significantly greater degree. Based on these data, it is apparent that spinal serotonin more effectively modulates antinociception against thermal pain, whereas spinal norepinephrine more effectively modulates antinociception against mechanical pain in both neonatal and more mature animals.

The final hypothesis tested in this experiment was that put forth by Giordano and Barr in 1987. They proposed that analgesia in response to systemic morphine develops in a rostral to caudal direction along an animals body due to the postnatal development of descending projections from nuclei in the RVM to the spinal cord. If this hypothesis is correct, analgesia resulting from morphine or glutamate administration to the PAG should also develop in a rostral to caudal direction since they are subserved by this spinopetal system. The findings of Experiment 1 lend support to this hypothesis, as GPA and OA are both noted in the forepaw and hindpaw at earlier ages than in the tail.

A model for the development of analgesia mediated by the PAG: Possible mechanisms

The data obtained in this thesis suggests a model for the development of analgesia mediated by the PAG. It is apparent based on these experiments that cells within and efferent to the PAG are markedly different in 3, 10 and 14 day old rat pups. Based on the pattern of analgesia that is seen at these ages we can hypothesize much about cells and receptor types that might be mature and function at various developmental stages. These hypotheses must eventually be tested further using tract tracing and autoradiographic techniques.

Figure 11 shows a hypothetical model for the mechanisms underlying analgesia from the PAG in 3 day old pups. At this age, glutamate responsive neurons are thought to be located exclusively within the dorsal aspect of the PAG and morphine responsive cells are found only within the ventral PAG. It is likely that the subtype of glutamate receptor found in the dorsal PAG at this age is the NMDA type, since this subtype has been found to develop earliest in other brain areas. The subtype of morphine receptors in the ventral PAG are probably the μ type as these receptors have been repeatedly localized in ventral and

ventrolateral PAG regions. In addition to the differential distribution of receptor types in the neonatal PAG, there also appear to be different projections from cells within the dorsal and ventral PAG to RVM nuclei. This is proposed because of the stimulus specificity of the analgesia produced by morphine and glutamate administration to ventral and dorsal PAG sites respectively. Descending serotonergic systems are thought to underlie analgesia against thermal noxious stimuli, whereas descending noradrenergic systems are thought to modulate analgesia against mechanical noxious stimuli. Thus it is likely that glutamate sensitive cells in the dorsal PAG project to excitatory interneurons within the PAG that in turn send excitatory projections somewhat exclusively to noradrenergic reticular nuclei. Morphine sensitive cells in the ventral PAG project to inhibitory interneurons within the PAG that in turn project somewhat exclusively to serotonergic raphae nuclei. The net effect of the morphine system is to release cells in the RVM from tonic inhibitory control. Moreover at this early stage projections from the RVM to the dorsal horn of the spinal cord are not yet fully mature. These projections are thought to reach rostral portions of the spinal cord that innervate the forepaws but not more caudal portions of the spinal cord that innervate the hindpaw and the tail. This is the proposed reason that analgesia is seen only in the forepaw of 3 day old pups.

In the 10 day old rat pup, a similar model for the cellular components of the PAG remains. However at this age the projections from the RVM to the the dorsal horn of the spinal cord have matured to the extent that they now reach more caudal levels of the spinal cord such as those that innervate the hindpaw. Therefore, in 10 day old pups analgesia is noted in the forepaw and the hindpaw. A schematic model for the cellular components of and projections from the PAG related to analgesia in 10 day old pups is found in Figure 12.

In the 14 day old rat pup, the cytology and synaptology of the PAG is markedly more complex. At this age, either glutamate or morphine will produce analgesia whether injected into dorsal or ventral PAG sites. Furthermore, both compounds produce analgesia against both types of noxious stimuli at this age. The appearance of glutamate responsive cells in the ventral PAG and morphine responsive cells in the dorsal PAG requires an expansion of the models set forth for previous ages.

A hypothetical model for the mediation of morphine-induced analgesia by the PAG in 14 day old rat pups is shown in Figure 13. Cells with μ receptors continue to funtion within the ventral aspect of the PAG. However, the projections from these cells now extend to nuclei within the reticular formation as well as to the NRM. This is reflected by the ability

of morphine to produce analgesia against both thermal and mechanical stimulus types at this age. In addition, receptors that bind morphine are now present within the dorsal PAG. These receptor subtypes are thought to be distinct from the μ receptors that mediate morphine analgesia from ventral PAG sites for two reasons. First, they mature at a different rate than the μ receptor subtypes. This is thought to be why morphine has no analgesic effects when given neonatally to dorsal PAG sites. In addition, morphine is a less potent agonist at these receptor sites than at those within the ventral PAG. This is evidenced by the fact that morphine analgesia never reached cutoff levels even when the high dose of morphine was given to dorsal PAG sites. Although it is not know for certain which type of opioid receptor may be functioning within the dorsal PAG, a good candidate for this receptor type is the κ_3 receptor noted by Gistrak et al. (1988). This opioid receptor subtype is known to be present in rat brain and to bind morphine with less affinity than the μ receptor subtype. Therefore we propose the late development of cells with κ_3 receptors in the dorsal PAG that send inhibitory projections to inhibitory interneurons within the PAG. The net effect is to release cells in the RVM from tonic inhibitiory control. Analgesia is noted in all limbs tested at this age, indicating that spinopetal projections from the RVM to the dorsal horn have matured.

A model for glutamate sensitive systems in the PAG of 14 day old pups is outlined in Figure 14. Cells with NMDA receptors continue to be present and functional within the dorsal aspect of the PAG. However, the projections of these cells are more extensive than they were at previous ages, and now include the NRM as well as the reticular nuclei as their sites of termination. Cells with glutamate receptors are now present in the ventral PAG as well as in the dorsal PAG. The receptor subtypes for glutamate found in the ventral PAG are probably not the same as those found in the dorsal PAG on the basis of their differential ontogeny. Therefore, we propose that one of the other glutamate receptor subtypes such as kainic or quisqualic acid receptors may mediate glutamate produced analgesia from ventral PAG regions. We propose that glutamate sensitive cells in the PAG send excitatory projections excitatory interneurons within the PAG. These interneurons stimulate serotonergic and noradrenergic nuclei within the RVM thereby producing analgesia. Furthermore, projections from the RVM to the spinal cord are thought to be fully developed at 14 days of age. For this reason, analgesia is now noted in the forepaw, hindpaw and tail.

The synaptology, neuroanatomy, and functions of the PAG are quite complex. By examining the analgesia mediated by this area during development, this thesis elucidated

several aspects of this structure that are not as apparent in the adult animal, and that may be useful for directing future research. Specifically, the PAG can be functionally divided into a dorsal and a ventral region with regard to analgesia. Glutamate and morphine both produced analgesia when administered to the PAG but distinct differences in the optimal site of administration of these compounds within the PAG as well as their ability to produce analgesia against thermal and mechanical stimuli were noted. Despite these differences, OA and GPA both appeared to be mediated by bulbospinal monoamine systems that project form the PAG to nuclei in the RVM and from these nuclei to the dorsal horn of the spinal cord by way of the DLF. Stimulation of the PAG with glutamate or morphine produces analgesia by causing the release of spinal serotonin and norepinephrine into the spinal cord. Antagonizing the effects of either of these spinal monoamines will antagonize GPA and OA from the PAG. Serotonergic antagonism was shown to attenuate analgesia against the thermal stimulus to a greater degree whereas noradrenergic antagonism more potently attenuated analgesia against the mechanical stimulus. In addition both GPA and OA from the PAG developed in a rostral to caudal direction, presumably mirroring the ontogeny of fibers fom the RVM that synapse at different levels of the spinal cord. The precise mechanisms that underlie the differential maturation of GPA and OA are not known at the present time. However, a hypothetical model has been proposed that should be the focus of future research.

Figure 11. Schematic representation of cells within and efferents from the PAG that mediate analgesia produced by morphine and glutamate in 3 day old rat pups. At this age, glutamate responsive cells are located exclusively within the dorsal PAG and morphine responsive cells are located exclusively within the ventral PAG. The NMDA receptor subtype is thought to mediate analgesia at this age since this receptor subtype develops earlier than other types of glutamate receptors. Moreover, glutamate produces analgesia only against mechanical noxious stimuli whereas morphine produces analgesia only against thermal nociceptive stimuli. This suggests that efferents from the dorsal and ventral PAG differentially synapse on noradrenergic and serotonergic nuclei within the rostral ventral medulla in the neonatal rat. In addition, antinociception following intraPAG treatments is restricted to the forepaw in 3 day old pups, indicating that the development of spinopetal pathways is incomplete at this early age.

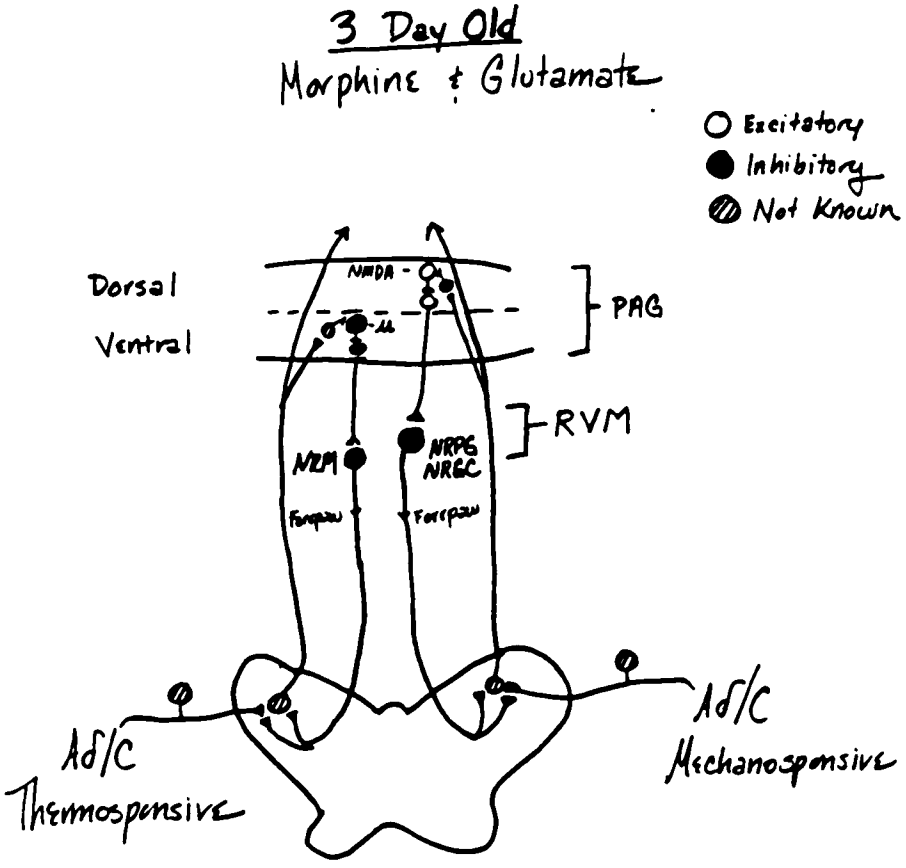

3 Day Old
Morphine & Glutamate
Excitatory
Inhibitory
Not Known
Dorsal
Ventral
NMDA
μ
PAG
RVM
NRM
NRPG
NRGC
Forepaw
Forepaw
Aδ/C
Thermosensitive
Aδ/C
Mechanosensitive

Figure 12. Schematic representation of cells within and efferents from the PAG that mediate analgesia produced by morphine and glutamate in 10 day old rat pups. At this age, glutamate responsive cells continue to be located exclusively within the dorsal PAG whereas morphine responsive cells are located exclusively within the ventral PAG. Glutamate-produced analgesia is still measureable against mechanical noxious stimuli and morphine-produced analgesia is only seen against thermal nociceptive stimuli suggesting a continued distinction between the termination sites of dorsal and ventral PAG efferents. In the 10 day old pup, antinociception is seen in the forepaw and the hindpaw. This suggests that bulbospinal efferent projections are more mature than they were in 3 day old pups, although they are not yet fully developed.

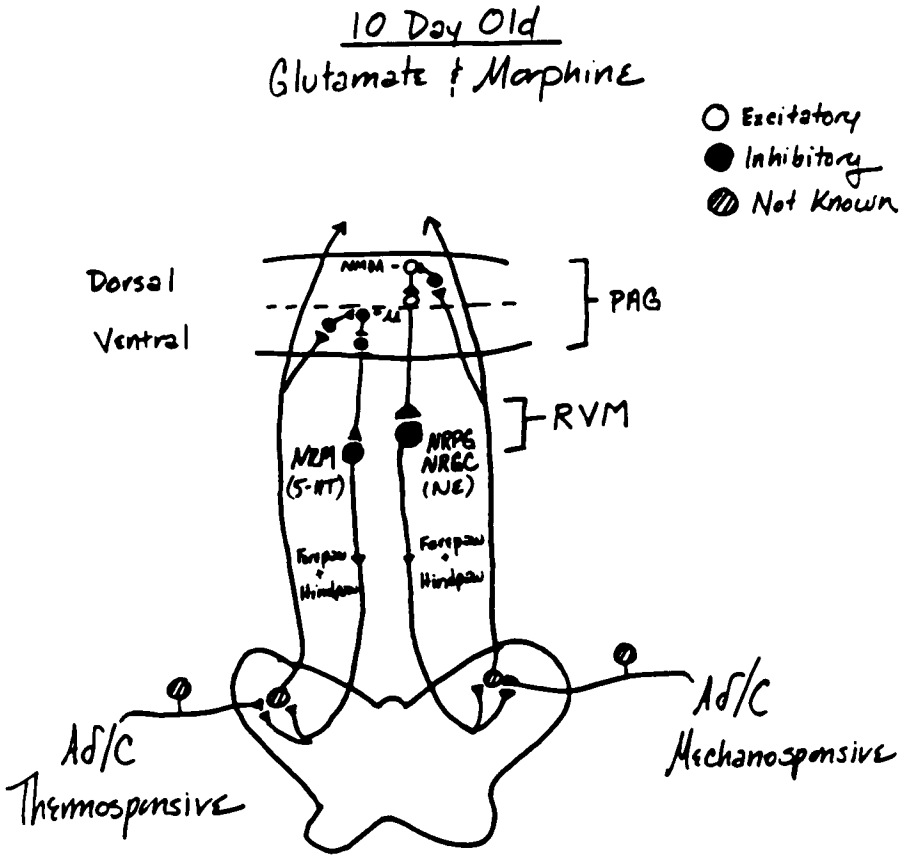

10 Day Old
Glutamate & Morphine
Excitatory
Inhibitory
Not Known
Dorsal
Ventral
NMDA
PAG
RVM
NRM
(5-HT)
NRPG
NRGC
(NE)
Aδ/C
Thermosensitive
Aδ/C
Mechanosensitive

Figure 13. Schematic representation of proposed cells and pathways that mediate morphine-induced analgesia in the 14 day old pup. At this age, morphine-sensitive cells are located in both the dorsal and the ventral PAG. This probably reflects the later development of a novel population of opioid receptors that mediate analgesia from dorsal PAG sites. These receptors are thought to be the κ_3 subtype based on the description put forth by Gistrak et al.(1989). At this age, morphine administered to the dorsal or the ventral PAG produced analgesia against both thermal and mechanical noxious stimuli. This finding suggests the addition of new projections from opioid sensitive cells in the PAG to noradrenergic nuclei witin the rostral ventral medulla to those present in the 10 day old rat pup. In the 14 day old pup, morphine-induced analgesia was seen in the forepaw, hindpaw and tail indicating that bulbospinal projections are fully mature at this age.

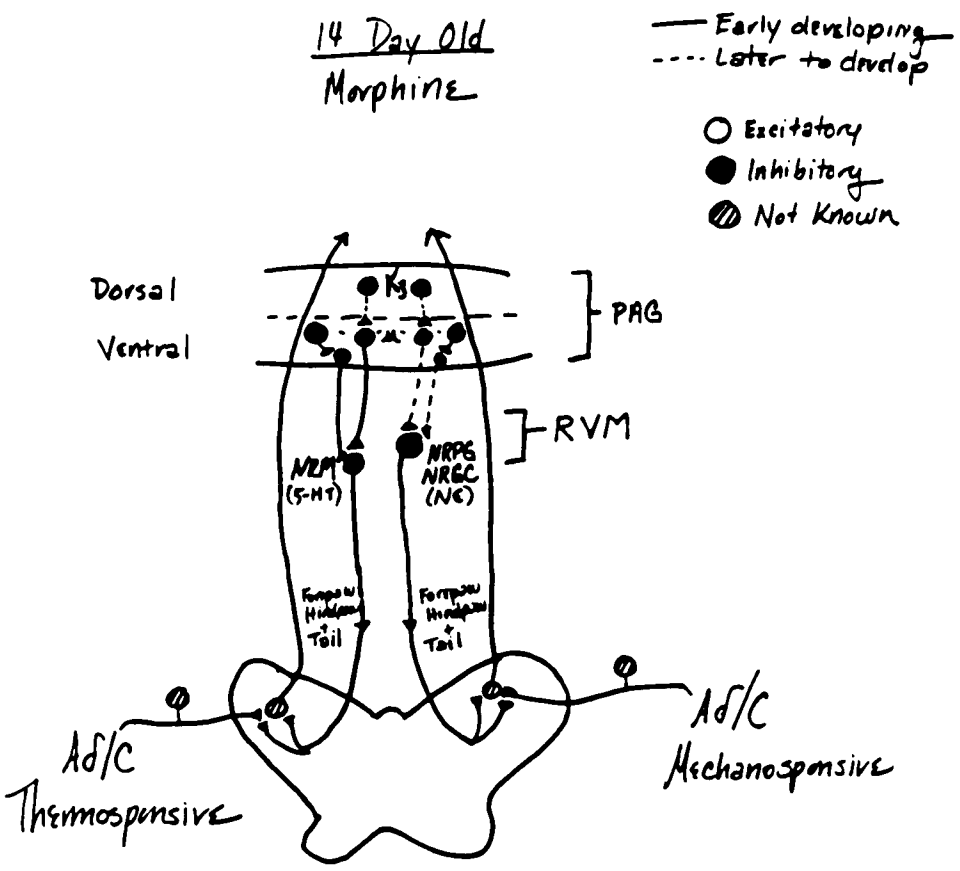
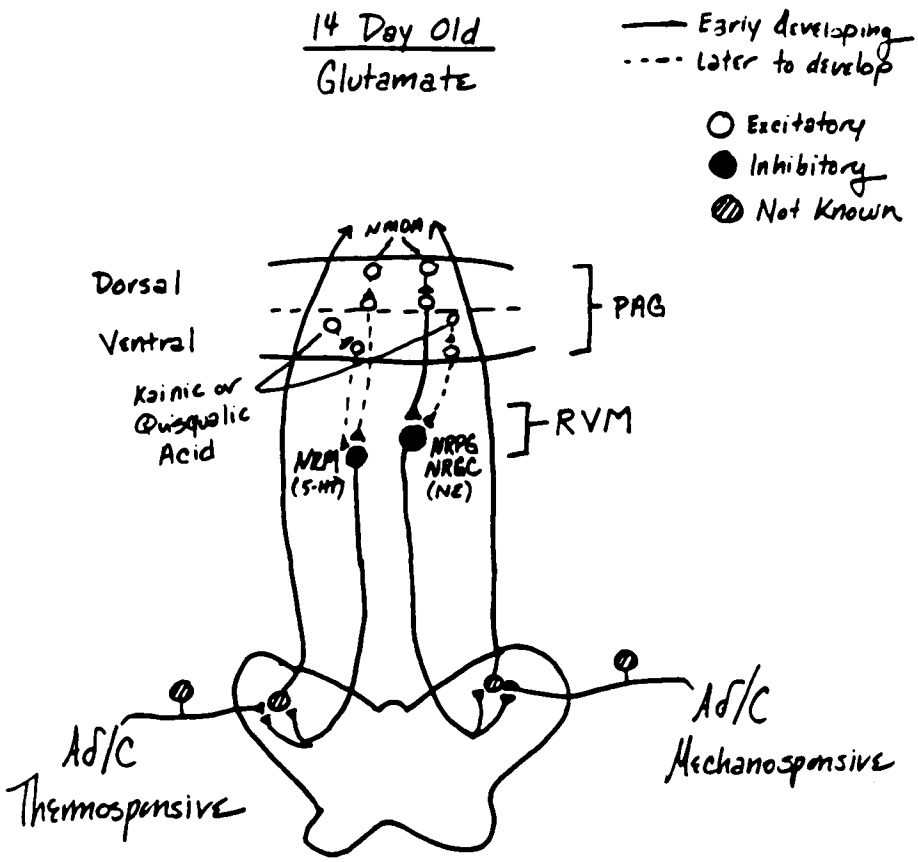

14 Day Old
Morphine
Early developing
Later to develop
Excitatory
Inhibitory
Not Known
Dorsal
Ventral
PAG
RVM
NRPG
NRGC
(NC)
Forepaw
Hindpaw
Tail
Aδ/C
Thermosensitive
Aδ/C
Mechanosensitive

Figure 14. Schematic representation of proposed cells and pathways that mediate glutamate-produced analgesia in the 14 day old pup. Glutamate-sensitive cells are located in both the dorsal and the ventral PAG at this age. A later developing population of glutamate receptors in the ventral PAG is thought to underlie this finding. This new receptor population could consist of the kainic acid or quisqualic acid receptor subtype or of both receptor subtypes. Glutamate administered to the dorsal or the ventral PAG in 14 day old pups produced analgesia against both thermal and mechanical noxious stimuli. Therefore, the development of new projections from glutamate responsive cells in the PAG to serotonergic nuclei witin the rostral ventral medulla thas been proposed. The finding that glutamate-produced analgesia was present in the forepaw, hindpaw and tail supports the hypothesis that descending bulbospinal systems are fully mature at this age.


14 Day Old
Glutamate
Early developing
later to develop
Excitatory
Inhibitory
Not Known
NMDA
Dorsal
Ventral
PAG
Kainic or Quisqualic Acid
RVM
NRM (5-HT)
NRPG NRGC (NE)
Aδ/C Thermosensitive
Aδ/C Mechanosensitive

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