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The pharmaco-ontogeny of spinal noradrenergic receptor systems mediating behavioral analgesia in the rat

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The Pharmaco-ontogeny of Spinal Noradrenergic Receptor Systems Mediating Behavioral Analgesia in the Rat

by

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Abstract

The Pharmaco-ontogeny of Spinal Noradrenergic Receptor Mediated Systems Mediating Behavioral Analgesia in the Rat

by

Harry E. Hughes

Advisor: Gordon A. Barr, Ph.D.

Behaviorally defined analgesia as measured by different pain assessment tests has been demonstrated to be under descending noradrenergic control. Peak noradrenergic receptor development in rat spinal cord occurs around 12 days of postnatal life. The first part of this thesis examined the development of analgesia produced by spinally applied noradrenergic agonists. The extent to which these drugs modulate pain information evoked by a thermal vs mechanical stimulus in the infant rat was also addressed. Intrathecal norepinephrine produced analgesia that was more pronounced against a mechanical than thermal stimulus and more pronounced in 10 day olds than 3 days olds. The α_2 receptor agonist clonidine produced a dose-dependent analgesia that first appeared at 7 days of age when tested with a thermal stimulus and 3 days of age when tested with a mechanical stimulus. The peak analgesic effect of clonidine occurred at 10 days of age. In contrast, the α_1 agonist phenylephrine was without analgesic effects. This developmental profile of behavioral analgesia correlates with the ontogeny of noradrenergic receptor activity in the spinal cord. The finding that intrathecal norepinephrine produced a more pronounced analgesia against a mechanical rather than thermal stimulus in the adult is supported by our investigation in the infant rat. The second part of this thesis examined the characteristics of opioid-noradrenergic interactions in modulating pain responses in the developing rat. Intrathecal injection of the α-antagonist phentolamine decreased analgesia produced by intraventricular morphine for a mechanical but not thermal stimulus. No difference between 3 and 10

day olds was observed in this effect. In contrast, phentolamine did not alter response latencies elevated by intrathecal morphine. The pharmacologic profile which characterizes opioid-noradrenergic systems for pain modulation in adult rats appears to be established early in development.

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This thesis contains 18 pages of listed references, almost all of whom I've never personally met. It is ironic that the names of those who have intimately assisted, guided and supported me through this effort are relegated to a single page. A full chapter seems appropriate. Thanks first of all to Professor Gordon Barr, my advisor and friend, for his patience and guidance. Shaping me into something resembling a scientist was not the easiest task that he has ever been called upon to do. The remaining members of my dissertation committee, Doctors Philip Zeigler, Gerald Turkewitz, Richard Bodnar and Alan Gintzler all deserve credit for contributing to the process.

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General Introduction

This thesis investigates the development of a behavior, analgesia, that has been demonstrated in studies with adult animals to be mediated in part by neurons that project from pontine and medullary nuclei to the spinal dorsal horn, and that contain norepinephrine (NE) as a neurotransmitter. Aspects of these neural pathways that contribute to the modulation of afferent pain signals in the adult subject have been studied anatomically, physiologically, biochemically and pharmacologically. The extent to which spinal, noradrenergic receptor mediated analgesia emerges as a behavior and reorganizes over time has not yet been studied. It was the purpose of this thesis to initiate such an investigation by defining, measuring and comparing the behavioral effects of noradrenergic and opiate compounds applied directly to the spinal cord of awake, infant rats at different stages of development. Before reviewing the studies pertinent to NE related analgesia, an overview of events that have led to a fundamental understanding of pain as a neural phenomena is in order.

Four important developments have occurred over the last five decades that have helped characterize the neural mechanisms underlying the phenomena of pain (Perl, 1985). First, it became evident that specialized, peripheral sensory receptors exist in mammals for detecting pain-inducing stimuli. Activation by graded electrical stimulation applied to progressively larger subsets of an afferent nerve was compared to verbal reports of sensation (Heinbecker, Bishop and O'Leary, 1933). Sensations related to pain depended upon the activation of small unmyelinated and slowly conducting myelinated fibers. These afferents have been classified as C and Aδ fibers respectively according to a nomenclature proposed by physiologists that corresponded to a scheme originally designed for muscle afferents (Kelly, 1985). However, the argument for specialized pain receptors apart from other tactile sense organs was not always

unanimously accepted. Zotterman (1939) reported that both vigorous and gentle stimulation of the skin resulted in the activation of these slowly conducting neurons. Douglas and Ritchie (1957) subsequently demonstrated that weak, mechanical stimulation of the skin activated most of the small, unmyelinated cells in the cutaneous nerve of the cat. Furthermore, firing thresholds for individual unmyelinated fibers in response to a mechanical stimulus of varying intensity varied widely (Iggo, 1960). These studies, combined with observations from other single unit recordings showing that very few afferents actually demonstrated elevated thresholds characteristic of a selected responsiveness to noxious stimuli, supported a non-specificity of function for tactile receptors. Not until a less biased selection of afferents based on conduction speed rather than ease of receptor field identification was employed did single unit recordings reveal a population of slowly conducting myelinated neurons that responded selectively to tissue damaging, mechanical stimuli (Burgess and Perl, 1967). In addition, tracing studies using horseradish peroxidase have characterized the morphology and distribution of these high threshold mechanoreceptors (HTMs). The terminals of these cells were found ipsilaterally in laminae I, II, and V of the spinal dorsal horn with some extensions that crossed the midline, terminating in the contralateral lamina V (Light and Perl, 1979). The spinal and medullary dorsal horns are laminated structures whose layers have been assigned roman numerals by Rexed (1952) to correspond with names such as outer marginal layer or substantia gelatinosa. Further single unit recording studies have profiled subsets of myelinated afferents that selectively serve in various combinations as low threshold mechanoreceptors (LTMs), HTMs, thermoreceptors, and chemoreceptors (Bessou, Burgess, Perl and Taylor, 1971; Bessou and Perl, 1969; Kumazawa and Perl, 1977). Finally, human reports of pain in response to electrical stimulation of single HTM- but not LTM-like fibers (Konietzny, Perl, Trevino, Light and Hensel, 1981; Torebjork and Ochoa, 1983) provided strong evidence for the presence of specialized pain-signalling receptors.

Secondly, these nociceptors provide information that is modulated and transmitted to the brain by subsets of neural afferent fibers. Importantly, a significant amount of the selectivity reported at the sense organ is preserved in the organization of secondary afferents (Kolmodin and Skoglund, 1960; Christensen and Perl, 1970) and relevant thalamic nuclei (Guilbaud, Peschanski, Gautron and Binder, 1980; Peschanski, Guilbaud, Gautron and Besson, 1980; Casey and Morrow, 1983; Honda, Mense and Perl, 1983). Projection neurons and interneurons in the spinal and medullary dorsal horns that receive input from peripheral nociceptors are of two general populations (Price and Dubner, 1977; Willis and Coggeshall, 1978; Dubner and Bennet, 1983). The first group is located in lamina I of the dorsal horn and projects rostrally as the neospinothalamic tract (Trevino, Coulter, Maunz and Willis, 1974; Willis, Leonard and Kenshalo, 1978; Willis, Kenshalo and Leonard, 1979). These cells terminate in three nuclei of the posterior thalamus; the ventrobasal complex (Perl and Whitlock, 1961; Angel and Clarke, 1975; Harris, 1978), the ventrocaudal parvocellular nucleus (Bowsher, 1961; Applebaum, Leonard, Kenshalo, Martin and Willis, 1979; Boivie, 1979), and the posterior nuclear group (Poggio and Mountcastle, 1960; Guilbaud, Caille, Besson and Benelli, 1977). Neurons from the neospinothalamic tract respond electrophysiologically only to intense mechanical or mechanical and thermal stimuli but are unresponsive to touch (Willis, Trevino, Coulter and Maunz, 1974; Willis, Maunz, Foreman and Coulter, 1975; Foreman, Applebaum, Beall, Trevino and Willis, 1975; Hoffman, Dubner, Hayes and Medlin, 1981). These nociceptive-specific (NS) cells may be important for the fine localization of sharp pain on the body surface (Perl, 1984).

The second group of neurons is located more deeply in lamina V of the dorsal horn and projects rostrally as the phylogenetically older paleospinothalamic tract. A portion of these cells terminate in the nonspecific intralaminar nuclei of the thalamus (Casey, 1966; Dong, Ryu and Wagman, 1978; Peschanski, Guilbaud and Gautron, 1981)

although most project to the reticular formation of the brain stem. This latter population of cells is more appropriately termed the spinoreticular tract. Electrophysiologically, cells from these tracts respond maximally to intense mechanical and in some cases thermal stimuli, but respond also to hair movements and other weak mechanical stimuli (Mendell, 1966; Handwerker, Iggo and Zimmermann, 1975; Menetrey, Giesler and Besson, 1977; Price and Dubner, 1977). These multireceptive or wide-dynamic-range neurons receive input from myelinated, low-threshold mechanoreceptors as well as from nociceptors and may mediate more diffuse, chronic types of pain (Willis and Coggeshall, 1978, for review). Both pain-signalling pathways ascend in the anterolateral area of the lateral spinal column and are collectively termed the anterolateral system. Though primarily crossed in humans, ipsilateral projections do exist and are implicated in the return of pain in patients whose anterolateral fibers are surgically sectioned to relieve chronic pain (Kelly, 1985).

Thirdly, pain-signalling information en route to brain somatosensory cortex is controlled by descending activity from supraspinal nuclei. Central nervous system (CNS) sites from which focal electrical stimulation resulted in the inhibition of dorsal horn afferents excited by noxious stimuli include the sensorimotor cortex (Coulter, Maunz and Willis, 1974; Yezierski, Gerhart, Schrock and Willis, 1983), ventrobasal thalamus (Gerhart, Yezierski, Fang and Willis, 1983), hypothalamus (Carstens, Mackinnon and Guinan, 1982; Carstens, Fraunhoffer and Suberg, 1983), midbrain periaqueductal gray (PAG) and lateral midbrain reticular formation (Carstens, Klumpp and Zimmermann, 1980; Gebhart, Sandkuhler, Thalhammer and Zimmermann, 1983; Gerhart, Yezierski, Wilcox and Willis, 1984), medullary nucleus raphe magnus (NRM) and adjacent rostral ventral medulla (RVM) areas (Fields, Basbaum, Clanton and Anderson, 1977; Gerhart, Wilcox, Chung and Willis, 1981; Gebhart et al., 1983; Gray and Dostrovsky, 1983), and the caudal medullary lateral reticular nucleus (Morton,

Duggan and Zhaq, 1984). The pharmaco-ontogeny of a selective population of these descending cells that uses NE as a neurotransmitter represents the basis of this dissertation and will be treated in greater detail below. It is important to realize, however, that centrifugal influences on somatosensory circuits include excitatory as well as inhibitory activity (Gebhart, 1986) and probably serve to modify most modalities of sensation (Perl, 1985).

Fourthly, the modulation of pain related neural activity is mediated by a system whose functional cytochemistry is highly complex and is only beginning to be understood. Immunocytochemical and autoradiographic studies have revealed the presence of many neuroactive peptides in the spinal and medullary dorsal horns including substance P, vasoactive intestinal polypeptide, cholecystokinin, somatostatin, bombesin, gastrin, a calcitonin-gene-related peptide (Hokfelt, Johansson, Ljungdahl, Lundberg and Schultzberg, 1980; Fuxe, Agnati, McDonald, Locatelli, Hokfelt, Dalsgaard, Battistini, Yanaihara, Mutt and Cuello, 1983), neurotensin (Seybold and Elde, 1982; Difiglia, Aronin and Leeman, 1984), enkephalin (Glazer and Basbaum, 1981; Bennet, Ruda, Gobel and Dubner, 1982), and dynorphin (Cruz and Basbaum, 1985). The presence of non-peptide putative transmitters in the dorsal horn including gamma-amino-butyric acid (GABA) (Miyata and Otsuka, 1975; Patrick, McBride and Felten, 1983), serotonin (LaMotte and de Lanerolle, 1983), dopamine (Blessing and Chalmers, 1979; Demenge, Feuerstein, Mouchet and Guerin, 1981), and NE (Nygren and Olson, 1977; Westlund and Coulter, 1980) has been confirmed. These substances derive from primary afferents, intrinsic interneurons of the dorsal horn, or supraspinal systems and provide a rich basis for the modulation of projection neuron output (Basbaum, 1985). Understanding the functional significance of a sizeable array of neuroactive substances in a concentrated spinal area is made even more difficult by the acknowledged co-existence of

some of these substances in single neurons (Hokfelt, Ljungdahl, Steinbusch, Verhofstad, Nilsson, Brodin, Pernow and Goldstein, 1978).

These four developments towards understanding the neural mechanisms underlying nociception provide the basis for a putative pain system that permits examination of behavioral analgesia by pharmacological challenge and the use of appropriate assays.

Introduction to Experiment 1

Evidence for Descending Control of Pain-Signalling Afferents in General

Microinjections of morphine (Tsou and Jang, 1964; Herz, Albus, Meyts, Schubert and Teschemacher, 1970; Jacquet and Lajtha, 1974; Criswell, 1976; Yaksh, DuChateau and Rudy, 1976) and electrical stimulation (Reynolds, 1969; Mayer and Liebeskind, 1974) applied to PAG resulted in behaviorally defined analgesia in a number of mammalian species. PAG stimulation also inhibited activity of nociceptive-specific neurons in the spinal and medullary dorsal horns (Mayer and Price, 1976; Basbaum and Fields, 1978; Sessle, Hu, Dubner and Lucier, 1981). Electrical stimulation of medullary nuclei including NRM (Oliveras, Redjemi, Guilbaud and Besson, 1975; Oliveras, Guilbaud and Besson, 1979; Proudfit and Anderson, 1975; Oleson, Kirkpatrick and Goodman, 1980; Satoh, Akaike, Nakazawa and Takagi, 1980; Zorman, Hentall, Adams and Fields, 1981; Hammond and Yaksh, 1984) and the nucleus reticularis paragigantocellularis of the RVM (Akaike, Shibata, Satoh and Takagi, 1978; Satoh et al., 1980; Zorman et al., 1981; Hammond and Yaksh, 1984) increased behavioral response thresholds to noxious stimuli with little effect on responses to tactile stimuli. These findings suggest that pain-signalling information is modulated at spinal and supraspinal sites (Dubner and Bennet, 1983, for review).

There exists evidence that descending monoaminergic fibers project to and inhibit cells in the dorsal horn (Dickenson, Oliveras and Besson, 1979; Sessle et al., 1981). The extent to which these descending neurons directly inhibit primary afferents or mediate inhibition postsynaptically via enkephalinergic, spinal interneurons is unclear (see below). Neurotransmitters contained within brain cells that project to the spinal cord include norepinephrine (NE) (Dahlstrom and Fuxe, 1965; Westlund, Bowker, Ziegler

and Coulter,1982), dopamine (Skagerberg, Bjorklund, Lindvall and Schmidt, 1982), and serotonin (Dahlstrom and Fuxe, 1965; Bowker, Westlund, Sullivan and Coulter, 1982). A number of centrifugal fibers are peptidergic, containing as neurotransmitters enkephalin, Substance P, thyrotropin-releasing hormone (Hokfelt, Terenius, Kuypers and Dann, 1979; Bowker, Westlund, Sullivan, Wilber and Coulter, 1983), and cholecystokinin (Skirboll, Hokfelt, Dockray, Rehfeld, Brownstein and Cuello, 1983; Mantyh and Hunt, 1984) among others.

The Role of Norepinephrine in the Spinal Cord

Various lines of evidence suggest that inhibition of spinal dorsal horn activity and behaviorally defined analgesia are under descending noradrenergic control. Dorsal horn nociceptive neurons were inhibited following iontophoretically applied NE (Belcher, Ryall and Schaffner, 1978; Headley, Duggan and Griersmith, 1978) while intrathecally (spinally) applied NE produced a dose-dependent behavioral analgesia against a thermally noxious stimulus (Reddy, Maderdrut and Yaksh, 1980; Reddy and Yaksh, 1980). Analgesia and diminished activity of dorsal horn neurons followed electrical stimulation of the ventromedial medullary reticular formation. This effect was blocked by tetrabenazine, a NE depleter, and was re-established with L-DOPA, a NE precursor (Takagi, Doi and Kawasaki, 1975; Akaike et al., 1978). Electrical stimulation of central and peripheral pain related loci such as the magnocellular tegmentum, dorsolateral funiculus (DLF), or sciatic nerve resulted in the release of endogenous NE from the spinal cord (Tyce and Yaksh, 1981; Yaksh, Hammond and Tyce, 1981). Stimulation of the NRM also increased the efflux of NE into superfusates of the spinal cord (Hammond, Tyce and Yaksh, 1985). Systemic morphine (Shiomi and Takagi, 1976) and microinjection of morphine into the nucleus reticularis

paragigantocellularis (Kuraishi, Fukui, Shiomi, Akaike and Takagi, 1978) increased the concentration of the NE metabolite, normetanephrine in the spinal cord.

Anatomic evidence indicates that NE related antinociceptive effects result from changes in the activity of cells that descend to the cord from supraspinal sites. Cells containing NE derive from several pontine nuclei including the ventral locus coeruleus, the nucleus subcoeruleus, the medial and lateral parabrachial nuclei, and the nucleus of Kolliker-Fuse in the lateral pons, and were found to project to layers I and II of the dorsal horn via the DLF (Westlund and Coulter, 1980). More recently, immunohistochemistry of rat spinal cord using antibodies developed against three specific catecholamine synthesizing enzymes employed as comparative markers distinguished neurons that contained NE, epinephrine or dopamine (Mouchet, Manier, Dietl, Feuerstein, Berod, Arluison, Deneroy and Thibault, 1986). The peroxidase anti-peroxidase and indirect fluorescence techniques permitted the precise location and distribution of catecholamine perikarya and the nature of the neurotransmitter they contained. Somata containing NE were located most caudally in cell groups at the upper cervical region of the spinal axis. These cervical neurons most likely represent a caudal extension of noradrenergic nuclei from medullary sites. Therefore, the antinociceptive effects of spinally applied noradrenergic agonists are mediated by NE containing cells not intrinsic to the spinal cord.

Developmental Aspects of Descending Noradrenergic Neurons that Modulate Pain

While the pharmaco-dynamics of spinal antinociception have been well investigated in the adult, few studies have addressed the emergence of pain modulatory systems in the developing animal. The ontogeny of descending monoaminergic pathways that subserve analgesia has not been studied at all. Receptor ontogenesis and the extent to which spinal

circuitry emerges and reorganizes over time determine the kinds of studies needed to address the development of behavioral responses to noxious stimuli. Developmental studies of opiate induced analgesia have provided some information in this area. For instance, the prototypic κ-opiate receptor ligand, ketocyclazocine, systemically administered produced analgesia against a thermal stimulus at age 10 days while morphine (a μ-opiate receptor ligand) was without effect until age 14 days in rats (Barr, Paredes, Erickson and Zukin, 1986). This study established a role for the κ-binding site in thermal analgesia and distinguished its ontogenetic pattern from that of the μ–receptor. It has also been demonstrated that shock induced analgesia was less in 10 day old rats than in 28 day olds or 5-7 month olds. Naloxone increased analgesia in 10 day olds, completely blocked analgesia in 28 day olds, and partially blocked it in 5-7 month olds. These results suggest that an opioid system mediated analgesia in the 28 day old that could be sensitized by naloxone at 10 days of age but not later, and that yielded to a more slowly developing non-opioid system as the rat matured (Hamm and Knisely, 1984). Furthermore, short term isolation from the nest environment resulted in a naltrexone (an opiate antagonist) reversible, analgesic reaction to heat in 10 day old rats (Kehoe and Blass, 1986). Likewise, systemic morphine caused an analgesic response to heat in 6 day olds that was blocked by naloxone while pups deprived of maternal interaction and food for 24 hours demonstrated analgesia that was blocked by the serotonergic antagonist metergoline (Spear, Enters, Aswad and Louzan, 1985). Low affinity binding of opioid compounds in rat brain and spinal cord during the first two weeks of life remained constant as did the respiratory response to these compounds. High affinity opioid binding increased up to three fold during this time and was correlated with a forty fold increase in analgesia against a thermal stimulus (Pasternak, Zhang and Tecott, 1980; Zhang and Pasternak, 1981). Addressing the ontogenesis of opiate binding affinities has revealed different possible structure-activity relationships

for analgesia and respiratory functioning that might otherwise have been obscured by maturational processes.

Similarly, if spinal monoamines, or some aspect of their activity, serve to regulate pain at one point in development but not another, whether or not a prototypic noradrenergic receptor agonist exists for inducing analgesia in a developing organism may not be a useful question. Animals whose pain regulating systems are not yet complete provide a means for comparing the ontogeny of analgesic behavior with maturation of the neural substrate presumed to mediate that behavior. Simmons and Jones (1985) found that NE-stimulated cyclic adenosine 3', 5'-monophosphate (cyclic AMP) accumulation in whole rat spinal cord peaked at age 12 days and declined to adult levels at age 30 days. Regionally, such accumulation peaked at 10 days in cervical and thoracic cord, 15 days in lumbar cord, and 20 days in cerebral cortex. One goal of the present study was to determine whether analgesia produced by spinally applied NE paralleled the reported development of noradrenergic receptor activity.

The Role of the Noradrenergic α_2 Receptor in Modulating Pain Sensation

A fundamental concern of this thesis is the functional onset of that aspect of analgesia which is presumed to be mediated by the noradrenergic α_2 receptor. The importance of receptor sub-type involvement is discussed below. As is usually the case, most of the studies cited used adult animals as subjects. The disproportionately smaller pool of developmental studies that addresses the emergence of behavior in view of well defined neural substrates, in itself underscores the need for more work in this area (see below).

The initial division of noradrenergic receptors into α_1- and α_2-subtypes was based on anatomical considerations (U'Prichard, 1984). Presynaptic receptors located on the terminals of noradrenergic cells were associated with the regulation of NE synthesis (Starke, Endo and Taube, 1975; Taube, Starke and Borowski, 1977) and were relegated to the α_2-subtype. Postsynaptic receptors constituted the α_1-subtype. Differences in the potencies of various adrenergic agonists and antagonists also contributed to the division of receptor subtypes (Langer, 1974). This topographical classification became less useful following the discovery of an α_2 receptor type profile that was not located presynaptically. These receptors were found in non-neural tissue such as platelets (Jakobs, Saur and Schultz, 1976) and neuroblastoma cell lines in culture (Kahn, Mitrius and U'Prichard, 1982). Current classification of noradrenergic receptors has a biochemical basis in that α_1 receptors mediate effects through elevation of membrane phosphoinositide turnover and intracellular calcium metabolism (Fain and Garcia-Sainz, 1980; Charest, Blackmore, Berton and Exton, 1983) while α_2 receptors mediate effects by inhibiting membrane adenyl-cyclase activity (Limbird, 1981).

The distribution of noradrenergic receptors with respect to α_1- and 2-subtypes does not appear to be uniform in the spinal dorsal horn. The terminals of small, unmyelinated afferents are located primarily in layers I and II, a region that demonstrates a high density of α_2, but not α_1 receptor sites (Young and Kuhar, 1980; Dashwood, Fleetwood-Walker, Gilbey, Mitchell and Spyer, 1985). Although this segregation of α-receptor subtypes implies but by no means demonstrates, a separation of function, the spinal administration of noradrenergic agonists and antagonists has demonstrated a role for noradrenergic α_2 receptors but not noradrenergic α_1 or β-receptors in modulating incoming pain information. The noradrenergic α_2 agonist clonidine was found to have dose-dependent analgesic properties when applied to the spinal cord while pain thresholds were unchanged after spinal application of the

noradrenergic β-agonist isoproterenol. The noradrenergic β-antagonist propranolol had no effect on analgesia resulting from intrathecally applied NE while such analgesia was blocked by the noradrenergic α–antagonist phentolamine when applied spinally (Reddy et al., 1980). Howe, Wang and Yaksh (1983) demonstrated a rank ordering of intrathecally applied agonists on several analgesic measures that argue for the involvement of a noradrenergic α_2 receptor population. For instance, the noradrenergic α_2 antagonist yohimbine reduced the analgesic responses produced in a dose-dependent manner by the intrathecal injection of the noradrenergic agonist ST-91 on tests that employ a thermal stimulus. Yohimbine was shown to be ten times more potent in this effect than was the α_1 antagonist, prazosin.

Inhibition of wide-dynamic-range neurons following iontophoresis of NE in the dorsal horn has been described against both a thermal and mechanical stimulus. The noradrenergic α_2 selective antagonists yohimbine and idazoxan reduced the magnitude of this response while α_1 antagonists WB4101 and prazosin did not. Similarly, α_2 agonists clonidine and metaraminol mimicked the inhibitory effect of NE on these neurons while the α_1 agonist phenylephrine had no effect (Fleetwood-Walker, Mitchell, Hope, Molony and Iggo, 1985).

Intrathecally applied noradrenergic α_2 agonists resulted in a suppression of the withdrawal response to noxious stimuli without any noticeable disruption of motor function. Analgesic doses of spinally applied NE or α_2 agonists resulted in no impairment of placing, stepping, or pinna twitch reflexes in rats (Reddy et al., 1980; Reddy and Yaksh, 1980). However, in doses higher than those needed to produce analgesia against a thermal stimulus, these compounds produced hindlimb and tail flaccidity (Yaksh and Reddy, 1981; Howe et al., 1983). High doses of intrathecally applied α_1 agonists resulted in motor impairments that were topographically distinguishable from those produced by NE or α_2 agonists. These effects included a dose-

dependent hyperreflexia, clonic flexion of the hindlimbs, rigidity, and serpentine movements of the tail (Yaksh, 1986).

These findings demonstrate that different populations of receptor subtypes related to pain sensation can influence motor output in distinguishable ways. The extent to which α_1 and α_2 receptors modify input to other systems remains to be seen. The adrenergic systems involved in pain control are linked intricately with limbic, reticular, and spinal circuits of motor and autonomic activity (Garver and Sladek, Jr., 1975; Hoffman, Felton and Sladek, Jr., 1976; Ishikawa and Tanaka, 1977; Crutcher and Bingham, 1978). Delineation of the receptor subtype that modulates input to any neural system represents a refinement in our understanding of the biochemical mechanisms underlying the pathology of that system. Such understanding must precede rational advances in pain therapy. If pain sensation can be diminished by the administration of compounds selective for the α_2 receptor, any possible "side effects" which result from the administration of compounds that also activate α_1 receptors could be eliminated. My second goal was to describe pharmacologically the noradrenergic receptor sub-type(s) involved in spinal antinociception in the preweanling rat.

Mechanical vs Thermal Stimulus

Recent evidence suggests that the descending NE pathway plays a more important role in the modulation of pain information evoked by a mechanical rather than a thermal stimulus. The order of sensitivity of three behavioral assays to intrathecal NE in the production of analgesia was tail-pinch > tail-flick > hot-plate while that of serotonin was tail-flick > hot-plate > tail-pinch (Kuraishi, Hirota, Satoh and Takagi, 1985). The tail-flick assay (D'Amour and Smith, 1941), like the hot plate assay (Woolfe and MacDonald, 1944), represents a response to a thermally noxious stimulus.

Furthermore, depletion of spinal NE resulted in a decrease in the mechanical but not thermal analgesia of morphine (Kuraishi, Harada, Aratani, Satoh and Takagi, 1983). To date, no studies have addressed the differential effects of NE on specified thermal and mechanical nociceptors or projection neurons. However, the participation of different putative transmitters in modulating pain information in the same area of the dorsal horn suggests different functions for these transmitters. Our third goal was to determine the extent to which spinally applied NE modulates pain information evoked by a thermal vs mechanical stimulus in the developing rat.

The Use of Drugs as Tools to Investigate Behavior

According to Ruth Levine (1983), the use of drugs for medicinal purposes dates back at least to ancient Egypt. The Ebers Papyrus of 1550 B.C.E. indicates more than seven hundred remedies and offers a detailed description of their preparation and application for specified disorders. The use of drugs within the context of experimental pharmacology however began shortly after William Harvey discovered that blood circulates. The intravenous route of drug administration enabled experimenters like Robert Boyle in 1660 to demonstrate temporal associations between the administration of a compound and biological effects. The study of drug toxicity thus followed and resulted in the formulation of principles that formed the basis of modern pharmacology. In the eighteenth century, Felix Fontana suggested that compounds contain an *active principle* that acts upon specified parts of an organism to produce characteristic effects. This finding helped to establish the idea proposed by the physiologist Claude Bernard and others in the following century that a drug works at a specific *site of action* to produce biological effects. Paul Ehrlich further developed this idea of specificity at the molecular level by coining the term *receptor* to account for the high affinity of

antibodies for specific antigens. Today, it is generally recognized that receptors serve as the macromolecules with which many drugs interact in living tissue to produce their effects. In 1776, the doctoral dissertation of Peter John Andrews Daries was published showing a relationship between the amount of a drug administered and the magnitude of it's biological effect. This thesis served as a preamble to the *dose response curve* which represents the most essential means for determining whether or not a biological or behavioral response is a direct result of drug action. Through the principles of mass action, drugs generally produce effects that are graded according to the amount of drug administered. This dose response relationship is most often plotted as some response represented linearly on the ordinate against the log of the dose along the abscissa. Because the log-dose response is a sigmoid curve, about 30 to 70% of it is represented by a straight line. A series of parallel curves can thus be obtained for different drugs that work by the same receptor mechanism in producing their common effects. The curve for the compound that interacts most strongly with the receptor will be to the left of the others, as lower doses will be required to produce the same response. Drug *potency* can thus be determined. Drugs whose dose-response curves are parallel with respect to slope are presumed to achieve their effects through activation of the same receptor. It thus becomes possible to determine if a class of drugs achieves a specified effect through a common mechanism. For instance, if analgesic response curves for hydromorphine, morphine, codeine and aspirin were compared, it would become evident from the more gradual slope of the aspirin curve that it produces analgesia by some mechanism other than that for the opiates whose curves would be parallel.

Usually, drug-receptor interactions are investigated in isolated tissue systems so that the exact amount of drug reaching a homogeneous receptor population to produce a single response is known. Pharmacological studies of behavior are more complicated. Behavioral responses cannot always be quantified on a linear scale. A behavioral

response may produce effects on other behaviors which in turn alter the response being measured. Also, drugs may have different effects on different receptor populations in the CNS resulting in a complex cascade of behavioral consequences that cannot be easily teased apart. Competitive binding studies may indicate that a given agonist is highly selective for a certain type of receptor. Even if the agonist binds with no other receptor type, multiple behavioral effects might still occur if the receptor type for which the drug is selective is widely distributed in the CNS and is linked to systems that mediate components of various behaviors (Iverson and Iverson, 1975). In spite of these problems, drugs can be used as tools to study behavior when the following considerations are addressed; a) An appropriate behavioral assay must be implemented. The behavioral response under study must be quantifiable and relatively reliable with respect to such factors as latency, duration, force, frequency and topography. Sufficient evidence must exist to ensure that inferences drawn from the test are applicable to the behavior. If a reaction to pain represents the behavior under study, the measured response should be one that occurs in the presence of pain inducing stimuli only. b) The drug being used should be selective for the receptor population that it interacts with. The development of drug isomers that differ from each other only in their three dimensional, molecular arrangement in space has resulted in agents that have been demonstrated through competitive binding studies to be highly selective for different receptor subtypes. These drugs are capable of affecting change in target cells while minimizing interactions with receptors whose activation might result in changes in behavior other than the one under study. c) Drug should be administered in a precise enough way to limit the number of loci activated, and still permit investigation of the behavior in question. Drug administration techniques such as iontophoresis, intracerebral and intrathecal injections permit the application of extremely small amounts of drug to specified areas of the CNS. These techniques effectively minimize the activation of cell populations that contain drug-responsive receptors but that are not

relevant to the behavior under investigation. These considerations and the limitations this thesis brings to bear on them are discussed in greater detail in the following section and throughout this thesis where applicable.

The pharmacological assessment of spinal contributions to behavior depends on the ability to deliver small amounts of drug directly to the spinal cord of live animals. Yaksh and Ruddy (1976) have contributed the technique of intrathecal catheterization whereby polyethylene tubing was inserted subdurally from the cervical to the lumbar spinal cord of the rat. Later, a method was described in which drug was introduced spinally through a lumbar puncture in mice (Hylden and Wilcox, 1980). Fu and Dewey (1981) reported an infusion procedure whereby laminectomized mice were fitted with a curved 30 gauge hypodermic needle in the subarachnoid space. Drug was then introduced through polyethylene tubing attached to the needle. While effective in the adult rodent, these procedures invariably resulted in severe damage to the spinal cord when applied unmodified to infant rats in our lab. To overcome this, we have developed a method of intrathecal implantation and injection that permitted examination of spinally administered drug effects in the developing rat. These procedures are described in the general methods section of this thesis.

Assessing Pain Reactions in Nonhuman Animals

An interdisciplinary approach to pain research has yielded a wealth of pharmacologic, physiologic, and anatomic information on the spinal circuitry underlying the coding of pain sensation. Perhaps the most difficult area in this attempt to understand the totality of influences on the spinal mechanisms of pain transmission has been the behavioral assays used to assess pain reactions in nonhuman animals (Vierck, Jr., Greenspan, Ritz and Yeomans, 1986). In humans, pain is a subjective phenomena whose parameters are

accessed and defined by verbal reports that reflect large variability among individuals. In animal tests, only behavioral reactions following a presumably noxious stimulus are measured (Wood, 1984). Analgesia is particularly difficult to measure because it represents the absence, or at least reduction, of observable reactions to pain producing stimuli. Vierck and Cooper (1984) have extensively surveyed the interpretive problems inherent in measuring animal pain reactions. Much of what follows in this discussion represents an extension of the issues presented in their treatise.

Thermal stimulation permits precise control of stimulus magnitude at the point of contact. However, non-nociceptive thermoreceptors presumably are activated before nociceptors due to the relatively gradual heat gradient across living tissue. This raises the possibility that test trials are terminated by "warm" receptor activation prior to nociceptor discharge when measuring limb withdrawal latency. What is presumed to be an escape response may in fact be an avoidance behavior or a simple reflex in response to non-noxious, thermal input. Receptor distribution, skin thickness, and degree of vascularization vary along the tail length. The blood supply to the tail is well adapted to transfer heat (Rand, Burton and Ing, 1965) which could reduce the extent of nociceptor activation by thermal stimulation. In fact, when *skin* temperature was measured during test trials using a radiant heat source, tail-flick *thresholds* varied from 38^{o}C to 45^{o}C (skin temp.) (Jackson, 1952; Hardy, Jacobs and Meixner, 1953). This finding suggests that when tail-flicks occur before the skin temperature is raised to 45^{o}C or higher (irrespective of heat source temperature), responses to non-noxious input are being measured. Ideally, electrophysiological recordings from thermal nociceptors during conditions that mimic the test situation should be performed to determine if true nociception is involved in the tail-flick response.

Another concern with behavioral assays that measure pain responses is the degree to which response magnitude is graded in proportion to stimulus intensity. A response

indicative of pain should occur preferentially to levels of stimulus intensity known to excite nociceptors and should be influenced differentially by intensities that produce mild versus intense pain. Therefore, in order for the tail-flick response to qualify as a reaction to pain, it is not sufficient merely that skin temperature exceeds 45°C. Properties of the response such as latency, duration, force, and perhaps topography should change in ways that indicate a painful reaction as the stimulus temperature increases within the painful range. Adjunctive behaviors that signify pain such as vocalizations and squirming might also increase during intertrial periods as the stimulus intensifies. However, the extent to which these behaviors reflect changes in levels of arousal rather than pain cannot be readily determined.

Finally, the degree to which a pain reaction is modified pharmacologically must be distinguishable from the extent to which nonpainful reactions are altered by the drug treatment. If an appropriately administered compound results in attenuated reactivity to presumed pain and if the rat is not rendered motorically impaired, the treatment may still not represent a modification of nociceptive input. As mentioned in an earlier section of this thesis, the adrenergic pathways representing pain control are closely connected with limbic, reticular, motoric, and autonomic systems. Therefore, suppression of central pathways linked to emotion, arousal, movement, or motivation that is less than complete may still alter response topographies on a number of behaviors. Control procedures that permit the discrimination of pain modulation from other behavioral effects of drug treatment should be implemented. Studies using drugs that interact with neurotransmitters at multiple CNS sites are especially in need of such controls. Also, the peripheral effects of drug treatment may influence behavior that is presumed to be mediated centrally. If systemically administered NE alters the flow of blood to the tail, how might this effect influence tail withdrawal latencies in light of the findings by Rand (1965) discussed above?

The unequivocal identification and quantification of behavioral responses to pain sensation still await test procedures that take into account the issues raised in the preceding paragraphs. Although reactions to thermally noxious stimuli only have been discussed, similar problems exist when testing pain reactions to noxious electrical, mechanical, and chemical stimuli as well. What follows are several arguments which justify use of the test procedures described in the general methods section of this dissertation. It is not suggested that these arguments "solve" the problems associated with measuring pain reactions, but rather permit a tentative behavioral model for studying pain in view of these problems.

The following observations are based on pilot studies done in our lab. The total submergence of appendages in water does not permit measurement of skin temperature apart from water temperature. Nor does this procedure allow electrophysiological recordings from single thermal nociceptors. Consequentially, in order to investigate whether or not test trials were being terminated by "warm" receptor activation prior to nociceptor activation, baseline latencies for appendage withdrawal were measured twice, 30 min. apart, in each of a group of young rats, and compared. No difference in baseline latencies was evidenced. This finding suggests that a conditioned response to non-noxious thermal stimulation which signalled the onset of pain was not learned. If the appendage withdrawal response represented an avoidance behavior rather than an escape behavior, a reduction in response latency following the second baseline test would be expected. It is important that in the proposed test procedures each animal was tested only twice for each appendage. Also, other centrally mediated phenomena such as habituation and sensitization do not seem to be involved in altering response latencies when testing is separated by a 30 min. interval and occurs only twice.

As the results of Experiment 1 demonstrate below, properties of the measured response changed in ways that indicated a painful reaction as the stimulus intensity increased.

Response latencies in non-treated rats decreased progressively for forepaw, hindpaw, and tail at water temperatures that ranged from 42°C to 54°C. At a water temperature of 40°C, no withdrawal response was evoked indicating that "wetness" alone was not an important component of the stimulus for eliciting a response (see "Discussion" section below for more detail). The latency of the response, which represented the dependent variable in these experiments, appeared to be graded in proportion to stimulus intensity and was influenced differentially by intensities that produced mild versus intense pain.

The means by which compounds were administered minimized the involvement of multiple CNS sites which could potentially influence components of the measured response. Although pain modulating adrenergic circuitry is linked with central systems that mediate emotion, arousal, and motivation and with peripheral systems that mediate blood flow, intrathecal application effectively bypasses the systemic route which ultimately supplies these auxiliary systems with drug. Intrathecal studies using adult rats have shown that very little drug redistributes to brain or peripheral tissue, and then only after considerable delay (Yaksh and Rudy, 1976; LoPachin and Rudy, 1982; Schmauss, Hammond, Ochi and Yaksh, 1983). It is understood that designating a drug application technique as a control procedure for discriminating pain modulation from other behavioral effects constitutes an a priori assumption and is therefore less than ideal. In fact, NE iontophorized unto spinal motor neurons produced a suppression of their activity (Engberg and Ryall, 1966; Weight and Salmoiraghi, 1966). However, until a more fundamental understanding of pain-specific behaviors is achieved, methods for distinguishing painful from nonpainful reactions to stimuli following drug treatment are tentative. Limitations of the intrathecal technique when applied to neonatal rats are discussed elsewhere in this thesis.

Aim of Study

This study attempted to provide information on the functional ontogeny of that aspect of behavioral analgesia to which spinal NE contributes. Age differences in the spinal effects of exogenously applied NE and noradrenergic agonists and the ability of different receptor subtypes to mediate these effects were examined by pharmacological manipulation.

Essentially, a single drug delivery technique was used to apply an array of compounds in different doses directly to the spinal cord of preweanling rats. These compounds are known to selectively interact with noradrenergic receptors and to mediate analgesia when spinally applied to adult rats (Kuraishi, Harada and Takagi, 1979b; Reddy and Yaksh, 1980; Reddy et al., 1980; Howe et al., 1983). Based on the immunohistochemical study of Mouchet et al. (1986) cited above, it was assumed that spinal NE derives from the terminals of descending cells whose perikarya reside in supraspinal nuclei. The failure by very sensitive assays to detect NE-containing cells intrinsic to the spinal cord does not necessarily mean that such cells do not exist. In fact, clonidine applied systemically produced analgesia in spinalized mice suggesting a segmental role for the agonist (Spaulding, Venafro, Ma and Fielding, 1979). However, the best available evidence currently indicates a supraspinal, descending organization for noradrenergic cells.

More specifically, the objectives of Experiment 1 were: a) To determine whether analgesic responses to noxious stimuli following drug treatment can be reliably measured in the developing rat. b) To determine whether the ontogenetic pattern of analgesia produced by spinally applied NE agonists paralleled the reported development of noradrenergic receptor activity. c) To ascertain the extent to which spinally applied NE modulates pain information evoked by a thermal vs mechanical stimulus in the

developing rat. d) To pharmacologically describe the noradrenergic receptor subtype(s) involved in spinally mediated antinociception in the preweanling rat.

Materials and Methods

Subjects. Offspring of Long-Evans hooded rats (Rattus norvegicus) bred and reared in Hunter College colony were used as subjects. Pregnant rats were checked for newborn pups twice daily. Pups found during these checks were declared born on that date, considered day 0. Dams and their litters were housed in 40 X 20 X 24 cm plastic terraria in a temperature and humidity controlled room. The light/dark regimen was 12:12 hours. Food (Purina pellets 5001) and water were constantly available. Three days following birth, litters were culled to 12 pups and otherwise left undisturbed until time of surgery.

Catheter. Subjects aged 2, 6, 9 and/or 13 days were implanted with intrathecal catheters. The catheter consisted of a 4 cm length of Dow Corning Silastic medical grade tubing (.012 in ID, .025 in OD) into which a flushed 1 cm length of Spectra/Por HF membrane (dialysis tubing with a 5000 MWCO) was inserted halfway at one end. The dialysis tubing was fastened to the silastic by a speck of cyano acrylate applied at the interface of the two tubings. Just prior to implantation, the catheter was filled with saline to check for possible leaks at the interface and to provide the dialysis tubing with a measure of firmness. The entire catheter held 3 µl of fluid. The dialysis end of the catheter was dipped in Methylene Blue dye (Sigma Chemical Co., St. Louis, Mo.) to enhance visibility prior to implant.

Surgery. Subjects were anesthetized with methoxyflurane (Metophane) and the skin was incised just above the thoracic and lumbar vertebrae. The underlying muscle and

fascia were gently scraped away exposing vertebrae T8 to T11. A laminectomy was performed with care taken not to injure the underlying dura. Removal of the dorsal aspects of three vertebrae exposed about .5 cm of spinal cord. The spinal dura was then punctured slightly off midline by moving the tip of a curved 30 gauge hypodermic needle caudally in a plane parallel with the spinal axis until the tip pierced the dura. Approximately .25 cm of the needle tip was inserted into the subarachnoid space with a gentle upward pressure to prevent damage to neural tissue. Immediately upon removal of the needle, leakage of clear spinal fluid was observed. Holding the catheter with forceps at the dialysis/silastic interface, the dialysis tubing was completely inserted in a caudal direction into the subdural space. Slow and steady advancement of the catheter prevented crimping of the dialysis tubing. The angle of the catheter lay parallel to the dorsolateral surface of the cord. The inserted catheter tip lay visibly above the cord. The silastic portion of the catheter was anchored by one drop of cyano acrylate to the first whole vertebra rostral to the laminectomized area. The glue was permitted to dry before closing the incision to prevent adherence of the catheter to the overlying tissue. The free end of the catheter remained outside of the animal to permit microinjections of drug directly to the surface of the spinal cord. The entire implantation procedure required about 15 minutes. After surgery, subjects were incubated at 32^{o}C and housed individually in plastic containers to prevent dislodgement of the catheter by sibling contact. Nourishment was provided in the form of .5 gram of yogurt placed on the floor of each container overnight.

Injection. After twenty-four hours, pups from the same litter were injected with soluble compounds in 4 µl of saline. Litters containing any animal that as a result of surgery displayed hyporeflexia, clonic flexion of the hindlimbs, tremors or rigidity were excluded from the injection procedure. To inject, a 10 µl Hamilton syringe was filled with 7 µl of drug solution and inserted 0.25 cm into the exposed end of the

catheter. Three µl of solution were injected into the catheter to fill the dead space followed by a steady injection of 4 µl solution at the rate of one 1 µl per 15 seconds. A speck of cyano acrylate sealed the exposed end of the silastic to prevent the remaining solution in the catheter from seeping into the cord. Following test procedures (described below), each animal was intrathecally injected with 4 µl of Methylene Blue dye using the same procedure implemented for drug injection. After a period of time equal to the time elapsed between drug injection and testing, pups were killed by pentobarbital overdose, the dorsal aspects of all vertebrae removed exposing the spinal cord, brainstem and cerebellum and the spread of dye observed. Litters containing any animal that did not demonstrate a profuse spread of dye from the caudal cervical segments of the cord to the lumbar/sacral region were excluded from the data. Also excluded were litters containing animals that exhibited any supraspinal staining. Preliminary studies from our lab indicated that a fluid volume of 4 µl applied spinally to infant rats as described reliably resulted in a spread of dye along the spinal axis within the limits defined above.

The time elapsed between injection and testing was determined for each drug by consulting previous intrathecal studies and by performing a time course procedure whereby young rats were administered a single dose of drug and tested in the manner described below at regular intervals after injection. The time during which a maximum increase in the analgesic response occurred represented the post-injection period for that drug. For instance, 10 day old rats received 2 µg intrathecal NE and were tested for analgesia against a thermal and mechanical stimulus paired at 5 min. intervals over a 35 min. period. We found that withdrawal response latencies were longest for both stimulus types 15 min. following injection. Therefore, the post-injection period for intrathecally applied NE was 15 min. This procedure was used to determine the post-injection period for all analgesic drugs used in the current study.

Intravenous Control. During surgery, the rupture of small blood vessels surrounding the spinal cord is unavoidable. That test results might reflect the action of drug that reaches the brain via a systemic route was addressed. Briefly, 9 day old rats were implanted with cannulae in the jugular vein as described by Harms and Ojeda (1974) with slight modifications for preweanling animals. Twenty-four hours later, subjects were injected with either 100 µg of clonidine HCl in 4 µl saline or with vehicle only directly into the jugular vein. This dose of clonidine represented the highest dose used during the intrathecal drug studies. Subjects were then tested for analgesia using a thermal stimulus as described below.

Drug Tests. All drug tests were conducted blind with respect to dose including vehicle. For all experiments except clonidine, subjects were exposed to both a thermally and mechanically noxious stimulus. In the clonidine study, each subject received only one type of stimulus. Those animals receiving two types of stimuli were exposed to both algesic tests in a random order of presentation. The thermal test consisted of a tail withdrawal assay modified to include other appendages. This treatment was shown to be sensitive to opiates (Janssen, Niemegeers and Dony, 1963) and other agonist/antagonist analgesics (Cowen, 1974; Grotto and Sulman, 1967; Sewell and Spencer, 1976). Prior to (baseline) and following drug injection, the forepaw, hindpaw and tail were sequentially submerged in water maintained at a temperature of 47^{o}C. Only appendages contralateral to the side of the cord into which the catheter was inserted were tested. Limbs ipsilateral to the catheter injection site occasionally demonstrated some loss of motor function following surgery. The order of testing each limb was randomly determined. The latency to withdraw each appendage from the water was measured by an electric timer (Lafayette Instruments Co.) and was recorded.

Those animals tested with a mechanical stimulus were treated as follows: Prior to and following drug injection, a 64 gram weight with a .2 cm diameter flat surface was applied sequentially to the dorsal surface of the center of the forepaw, hindpaw and tail contralateral to the side of the cord into which the catheter was implanted. Contact by the .2 cm surface of the weight was made with the surface of each appendage before allowing the weight to be manually released. This "adaptation" procedure insured that withdrawal-like movements during test trials did not occur in response to non-noxious, tactile stimulation. The order of testing each limb was random. The latency to produce withdrawal-like movements of each appendage following release of the weight was timed and recorded. Once injected, each of the three appendages was tested only once per stimulus type.

For all intrathecal drug studies, breaths per minute and righting response latencies were recorded for each animal just prior to baseline testing and prior to experimental testing. In addition, pups 7 to 14 days old were required to grasp a vertical wire mesh screen and support themselves for 20 seconds. Three day olds straight from the nest were unable to perform this test. These procedures were used to monitor general arousal in subjects receiving different doses of drug.

Variable Intensity Tests in Non-treated Rats. In order to determine whether the behavioral responses measured during the testing procedures signified a reaction to pain, a stimulus intensity study was performed. Non-drugged rats aged 3, 7, 10 and 14 days were tested in the above described manner using a series of thermal and mechanical stimuli that increased progressively in intensity magnitude. The series of thermal stimuli consisted of nine trials that ranged from 38^{o}C to 54^{o}C while the mechanical series consisted of five trials that ranged from 46 to 76 grams. Each trial included the testing of all three appendages. Whether a subject received the thermal or mechanical series first was randomly determined.

Variable Intensity Tests in Treated Rats. In order to determine whether differences between thermal and mechanical responding were due to drug action and not to possible intensity differences between the two stimuli, a second stimulus intensity study was performed. Catheterized, 3 day old rats were intrathecally injected with a single dose (6.0 μg) of NE and tested in the above described manner using a series of thermal stimuli consisting of three trials that ranged from 42°C to 52°C. The mechanical series consisted of three trials that ranged from 56 to 72 grams. Each trial consisted of the testing of all three appendages. Whether a subject received the thermal or mechanical series first was randomly determined.

Histology. In order to determine the extent of damage routinely inflicted on the spinal cord by the surgical procedure, a histological examination of the catheter implantation site was performed. Three day old rats that had been implanted 24 hours prior (equivalent to the time of behavioral testing) were overdosed with chloral hydrate and perfused intracardially with 4% paraformaldehyde. The vertebral column containing the cord was removed and soaked for 48 hours in 30% sucrose. The section of the column that contained the implant was frozen and 30 mm coronal sections were cut on a cryostat and thaw mounted on subbed slides. These sections were stained with cresyl violet or the Bodian silver stain (Bodian, 1936; Russel, 1973).

Drugs. All drugs for intrathecal injections were freshly prepared in physiologic saline. The following drugs were used: Clonidine HCl, norepinephrine HCl and the noradrenergic α_1 agonist l-phenylephrine HCl (Sigma Chemical Co., St. Louis, Mo.).

Statistics. Litters were treated as experimental units (Denenberg, 1977). Dose, limb and age effects were compared by means of a three-way factorial analysis of variance for most experiments. No age effects were compared for subjects receiving phenylephrine as only one age group was tested. Repeated measures were done for

limb and dose as all three limbs tested were from the same animal while all four doses were administered to the same litter. Post-hoc tests for simple main effects (Kirk, 1968) were used when applicable to determine the significance of response differences in control animals exposed to a thermal vs mechanical stimulus.

Results

Stimulus Intensity Function in Non-treated Rats. Appendage withdrawal latencies in non-drugged, infant rats decreased as thermal and mechanical stimulus intensities increased, even within the noxious range. Figures 1 and 2 represent response latencies as a function of stimulus intensity at all four ages for thermal and mechanical tests respectively. Figure 1 indicates that no withdrawal responses occurred at 38°C, that little responding occurred at 40°C, but that response latencies sharply declined at about 42°C.

Similar findings were observed with the mechanical stimulus test. Figure 2 indicates that no withdrawal responses occurred at 46 grams, that little responding occurred at 56 grams, but that response latencies sharply declined at 61 grams. Latencies continued to diminish as stimulus weight increased to 66 and 76 grams. The purpose of this study was to obtain withdrawal response curves by increasing the intensity of two types of noxious stimuli to the appendages of infant rats at different ages. The overall shape of the curves were of interest while the significance of possible differences between individual means was not. Therefore, no statistical analysis was done. Three animals from separate litters were used for each age group with a roughly equal distribution of males and females across the study. The findings suggest that the appendage withdrawal response is a pain reaction that is graded in proportion to stimulus intensity for both thermal and mechanical tests.

Inflammation of the skin of tested appendages was observed at water temperatures in excess of about 52°C. Weights greater than 70 grams resulted in contusions and intense vocalizations. Temperatures lower than about 44°C and weights under 61 grams resulted in motor responses that could not readily be distinguished from general locomotor activity. This was observed even though response latencies at these lesser

Figure 1. Withdrawal response latencies as a function of thermal stimuli applied to different appendages in four age groups of rats. Each value represents the mean response latency of three rats. Testing procedures are described in the text.

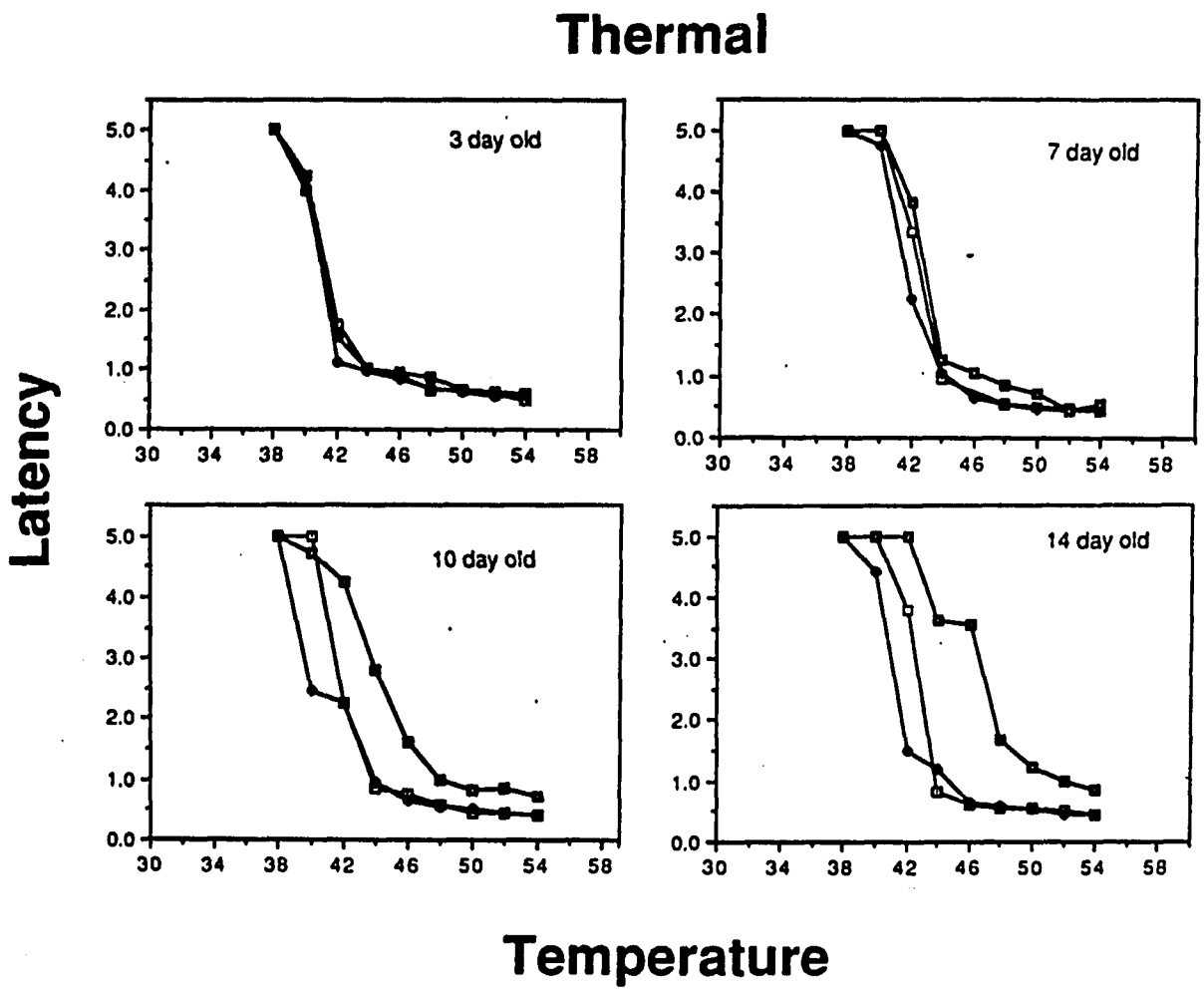
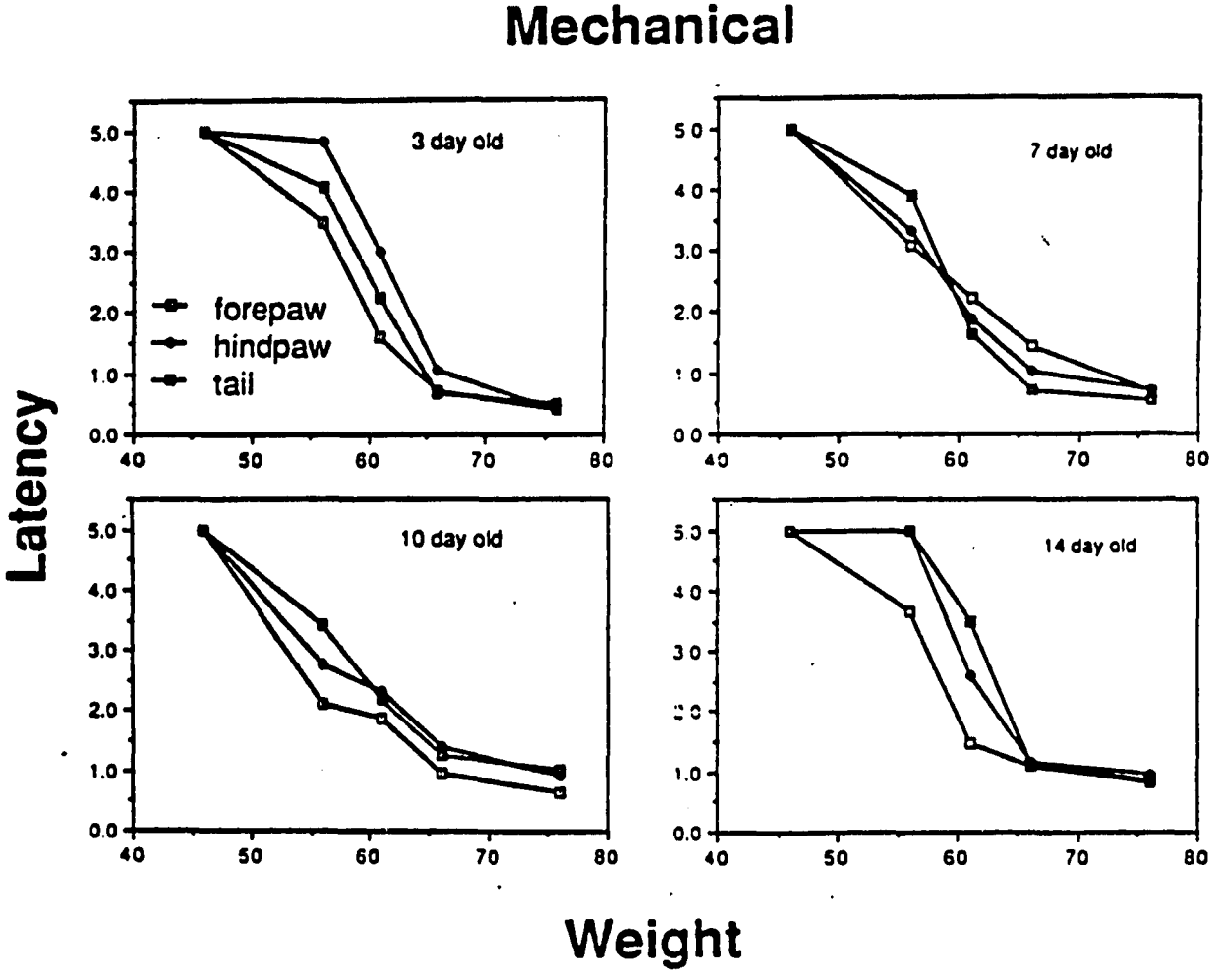

Thermal
Latency
3 day old
7 day old
10 day old
14 day old
5.0
4.0
3.0
2.0
1.0
0.0
30
34
38
42
46
50
54
58
Temperature

Figure 2. Withdrawal response latencies as a function of increased weight applied to different appendages in four age groups of rats. Each value represents the mean response latency of three rats. Testing procedures are described in the text.


Mechanical
Latency
Weight
3 day old
7 day old
10 day old
14 day old
forepaw
hindpaw
tail
5.0
4.0
3.0
2.0
1.0
0.0
40
50
60
70
80

intensities were sometimes comparable with those at higher intensities. Therefore, the stimulus intensities of 47°C and 64 grams used in the intrathecal drug studies were clearly within the noxious range and were sufficient to permit a reliable motor response topography without causing observable tissue trauma.

For all of the drug experiments with significant results, the onset of analgesia was rapid, reaching a peak in 15 min. and was not accompanied by any demonstrable changes in simple reflexes such as stepping or righting. Subjects were ambulatory and appeared free of sensory impairment. Following dye injection there was an even and reliable spread of dye from the cervical to lower lumbar segments. The rapid onset of the drug effect and the restriction of dye to caudal aspects of the cord suggested a role for spinal receptors.

Intravenous Control. Ten day old rats that received 100 μg clonidine directly through the jugular vein and were tested against a thermal stimulus demonstrated no differences in analgesic testing when compared to controls (data not shown). It is therefore unlikely that intrathecally applied drugs reach supraspinal sites by the vascular system in sufficient amounts to affect analgesic responding. Four litters were used with a roughly equal distribution of males and females per litter.

Histology. Figure 3 shows a photomicrograph of the Nissl stained cord with the implant. As can be seen, the implant disrupted the dorsal root ganglia on the side of the implant. In addition, there was often slight deformation of the cord beneath the cannula. We never noted damage to the other aspects of the cord including the contralateral side. Identical results were obtained using the Bodian stain (data not shown).

Norepinephrine. Intrathecal injections of NE produced a dose-dependent elevation in response latency on both thermal and mechanical tests. Figure 4 represents

Figure 3. Cresyl violet stained section of the spinal cord of a 4 day old rat pup that had been implanted with an intrathecal catheter 24 hours earlier. The section was cut at 24 microns through the vertebral column. The catheter is denoted by the "C" in the upper left portion of the spinal cord. Note that while there was disruption of the dorsal root ganglia and associated roots on the side of implant, there was no detectable damage contralateral to the catheter.

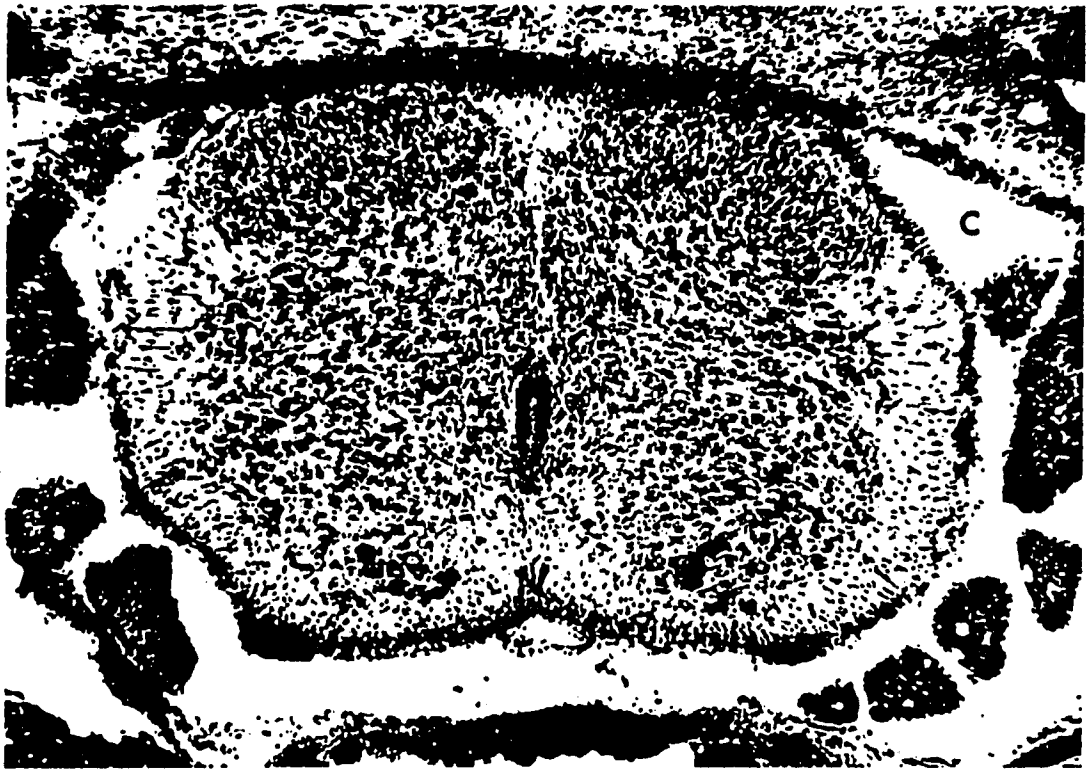
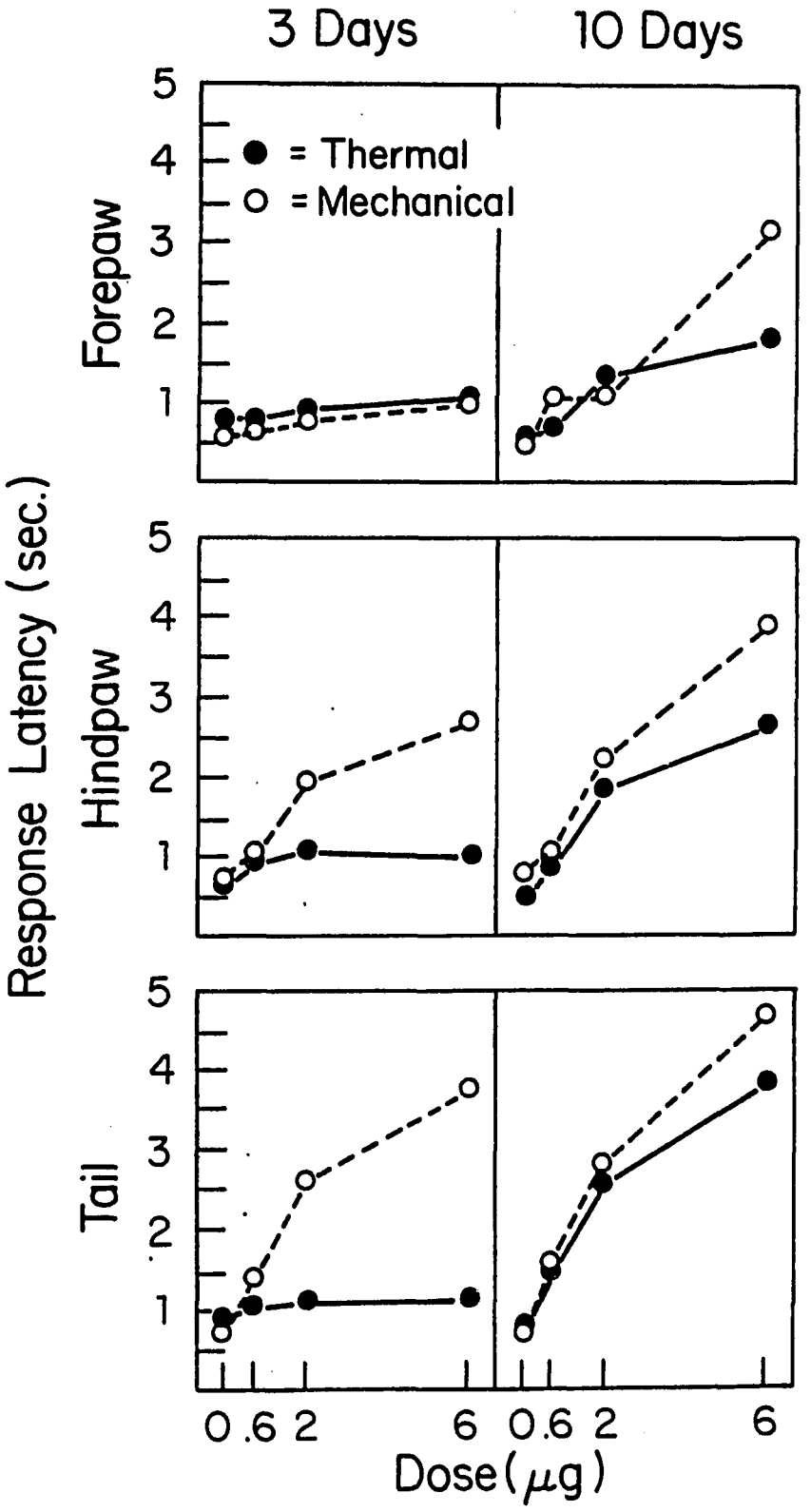

C

Figure 4. Effects of intrathecal NE on thermal and mechanical latencies for different appendages in two age groups of rats. Each value represents the mean withdrawal response latency of six rats. S.E.M.s for all means were less than ±0.55. Details of dosages and injection procedures are described in the text.

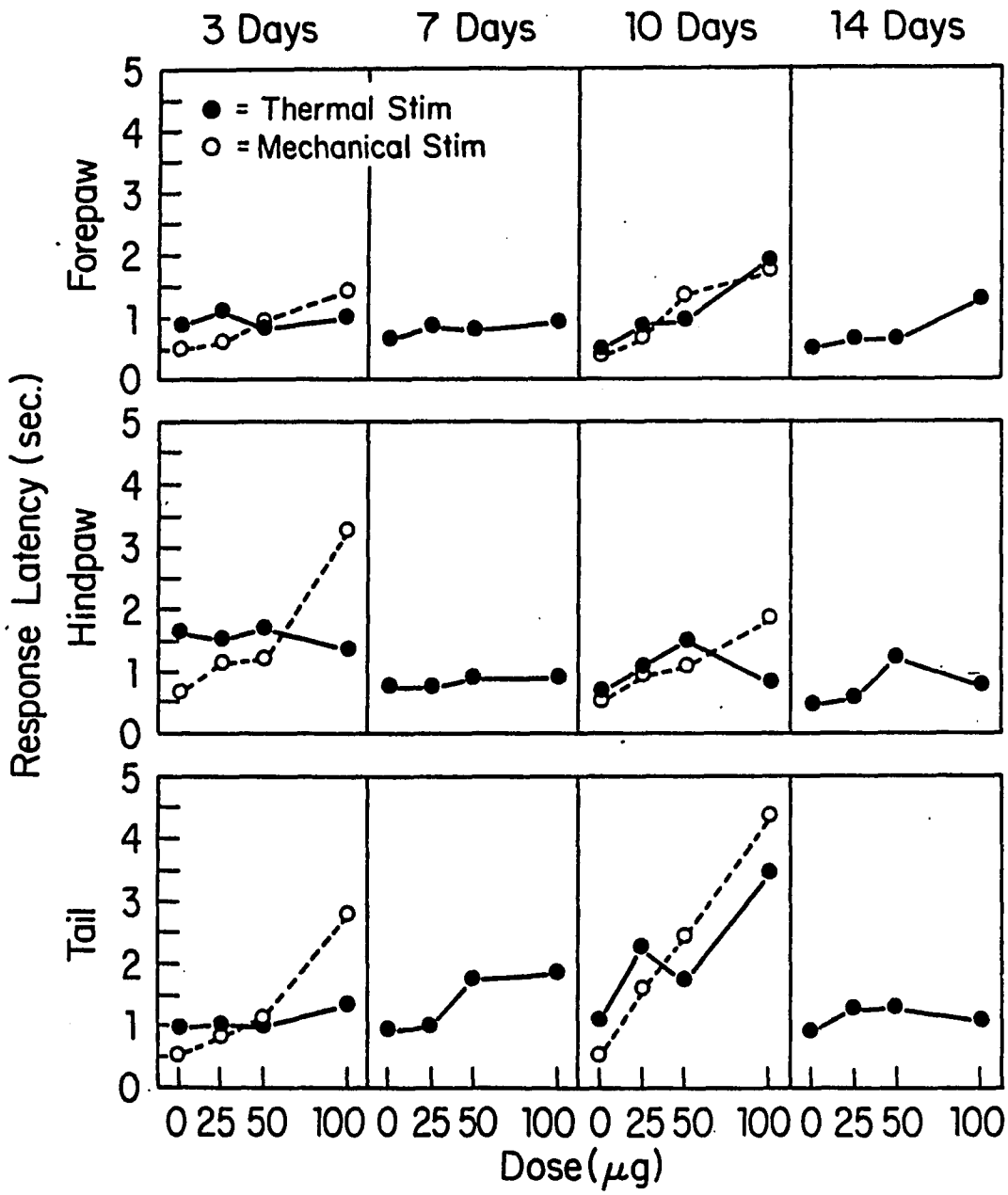

3 Days
10 Days
● = Thermal
○ = Mechanical
Forepaw
Hindpaw
Tail
Response Latency (sec.)
1
2
3
4
5
0.6
2
6
Dose (μg)

withdrawal response latency as a function of dose for each appendage in 3 and 10 day old rats. Inspection of Figure 4 shows that although NE induced analgesia against a mechanical stimulus occurred as early as 3 days postnatal life, 10 day old rats were more responsive to intrathecal NE than were 3 day olds [dose X age; $F, (3, 30) = 13.77, p < .01$]. NE produced longer response latencies against a mechanical stimulus than against a thermal stimulus as the drug dose increased, particularly in 3 day olds [dose X stimulus type; $F, (3, 30) = 16.96, p < .01$]. Also, the tail and hindpaw demonstrated significantly longer latencies overall than did the forepaw, [limb X dose; $F, (6, 60) = 9.20, p < .01$]. Differences between thermal and mechanical responding in the tail and hindpaw diminished as the animal matured. Six litters per age group were used with a roughly equal distribution of males and females per litter.

Clonidine. Intrathecal administration of clonidine produced a significant, dose-dependent elevation in response latency that was age dependent. Figure 5 represents the withdrawal response latency as a function of dose for each appendage within four age group of rats. The results of both thermal and mechanical tests are indicated for 3 and 10 day olds only. No significant difference in response latency among limbs was observed for the thermal tests [$F, (2, 44) = 1.81, p > .10$]. The thermal analgesic effect began at 7 days of age, peaked at 10 days and returned to previous values at 14 days of age. A dose by age analysis indicated that the thermal analgesic effect was significant [$F, (9, 66) = 2.62, p < .05$].

Against a mechanically noxious stimulus, intrathecal clonidine also resulted in analgesia. A dose by age analysis indicated that the analgesic effect was significant [$F, (3, 42) = 6.70, p < .001$]. Inspection of the graph shows that the mechanical analgesic effect began at 3 days of age, was significantly more pronounced in the hindlimb and tail [$F,(2, 28) = 4.11, p < .05$], and was greater than the thermal effect for the hindlimb and tail at both ages tested. Six litters were used for testing 3 day

Figure 5. Effects of intrathecal clonidine on thermal and/or mechanical latencies for different appendages in four age groups of rats. Each value represents the mean withdrawal response latency of five to eight rats. S.E.M.s for all means were less than ±0.86. Details of dosages and injection procedures are described in the text.


3 Days
7 Days
10 Days
14 Days
● = Thermal Stim
○ = Mechanical Stim
Forepaw
Hindpaw
Tail
Response Latency (sec.)
0
1
2
3
4
5
0 25 50 100
Dose (μg)

olds, eight for 7 day olds, seven for 10 and 14 day olds. Male and females were equally distributed within litters.

Phenylephrine. Figure 6 represents response latency as a function of dose for each appendage in 10 day old subjects. Intrathecal injection of phenylephrine had no effect on nociceptive responding regardless of stimulus type [$F, (3, 18) = 1.66, p > .10$]. No observable motor impairments followed the injection of small doses of the agonist. Spinal injection of 100 µg of the drug however, resulted in clonic flexion of the hindlimbs, rigidity and serpentine tail movements that were severe enough to preclude analgesic testing. The doses of 5 to 20 µg used in this study were comparable to those doses used in previous adult studies. Four litters were used with a roughly equal distribution of males and females per litter.

Stimulus Intensity Function in Treated Rats. Figure 7 represents response latencies expressed in maximum percent effect (MPE) as a function of stimulus intensity in 3 day old, NE-treated rats for thermal and mechanical tests respectively. MPE = [(Postdrug latency minus predrug latency)/(cut-off time minus predrug latency)] 100. MPE represents in this case the percentage of the maximum cutoff latency (5 seconds) achieved by a subject during a single trial. With the single exception of tail-flick latencies at a low temperature, NE produced no analgesic responding against a thermal stimulus at any intensity. Against a mechanical stimulus, NE resulted in elevated response latencies in the tail and hindpaw at mid to high intensity levels. These findings suggest that the difference in response curves between thermal and mechanical tests obtained in the drug studies described below are not due to a simple mismatch in the intensity of the two stimuli. The overall shape of the response curves were primarily of interest. Therefore, no statistical analysis was done for this study. Six litters were used with a roughly equal distribution of males and females per litter.

Figure 6. Effects of intrathecal phenylephrine on thermal and mechanical latencies for different appendages in 10 day old rats. Each value represents the mean withdrawal response latency of four rats. S.E.M.s for all means were less than ±0.21. Details of dosages and injection procedures are described in the text.

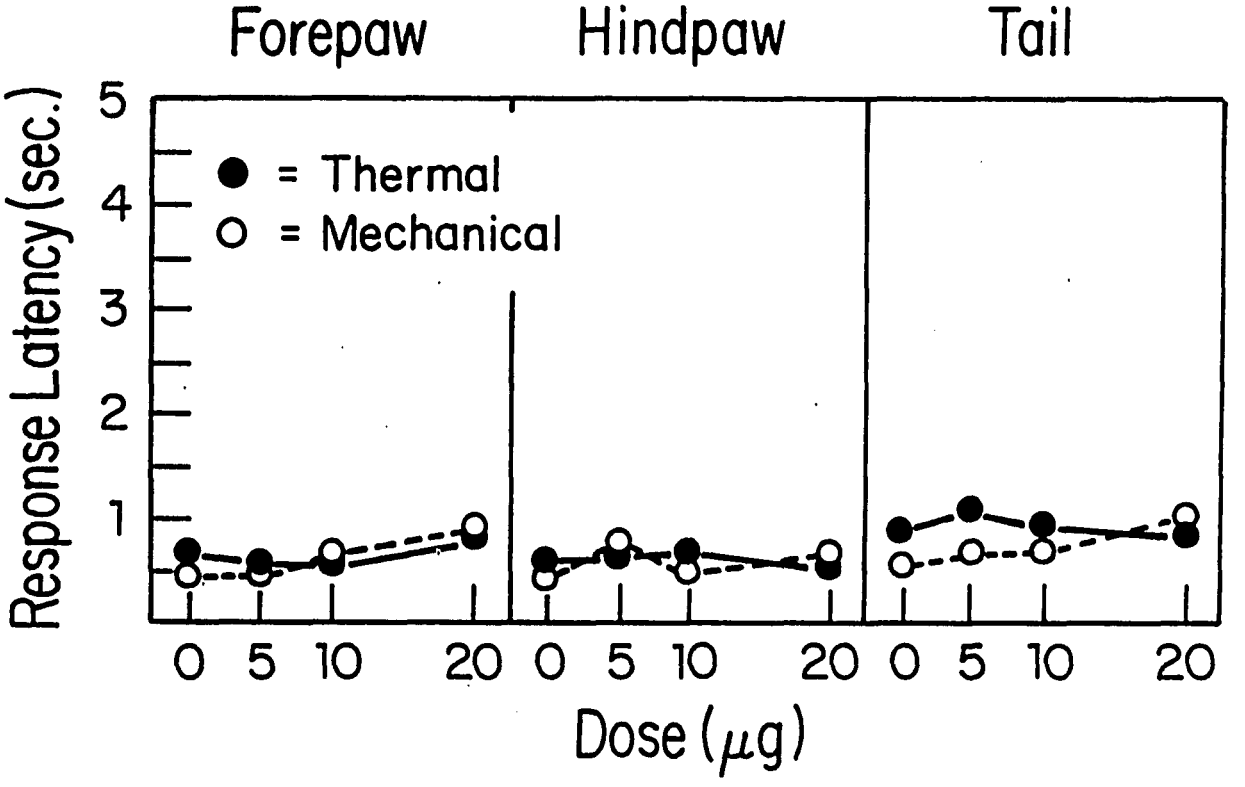
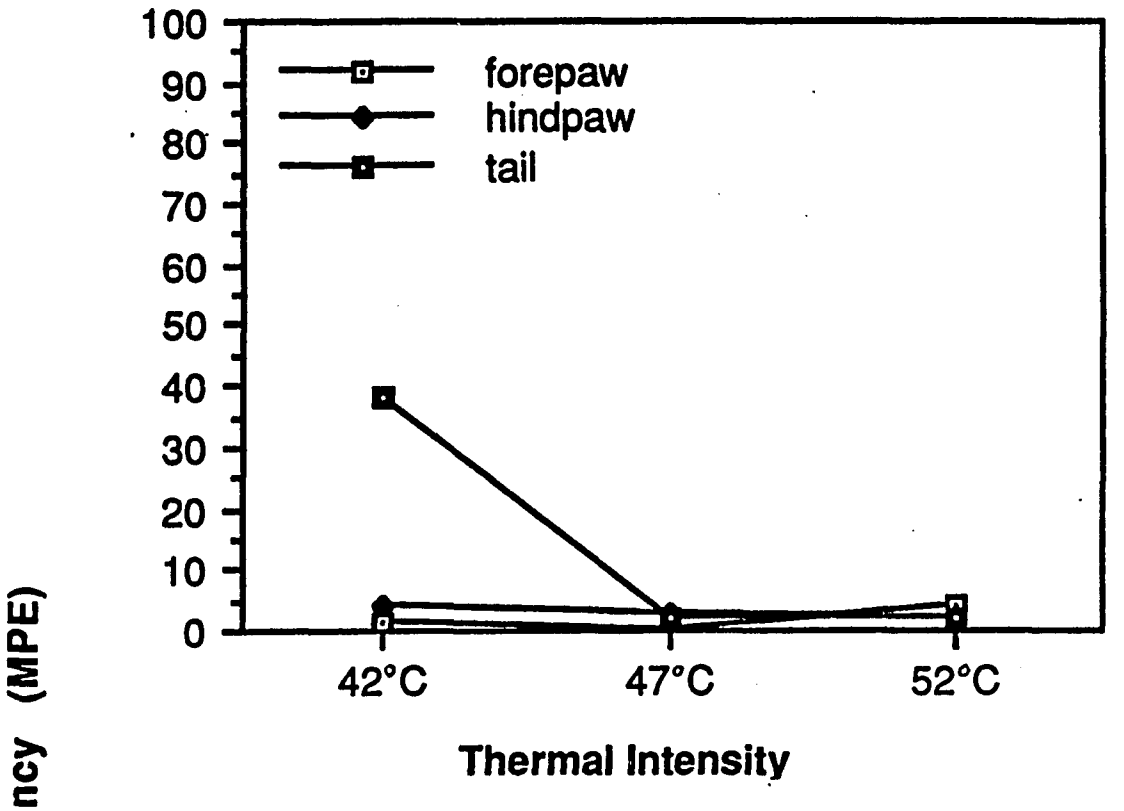
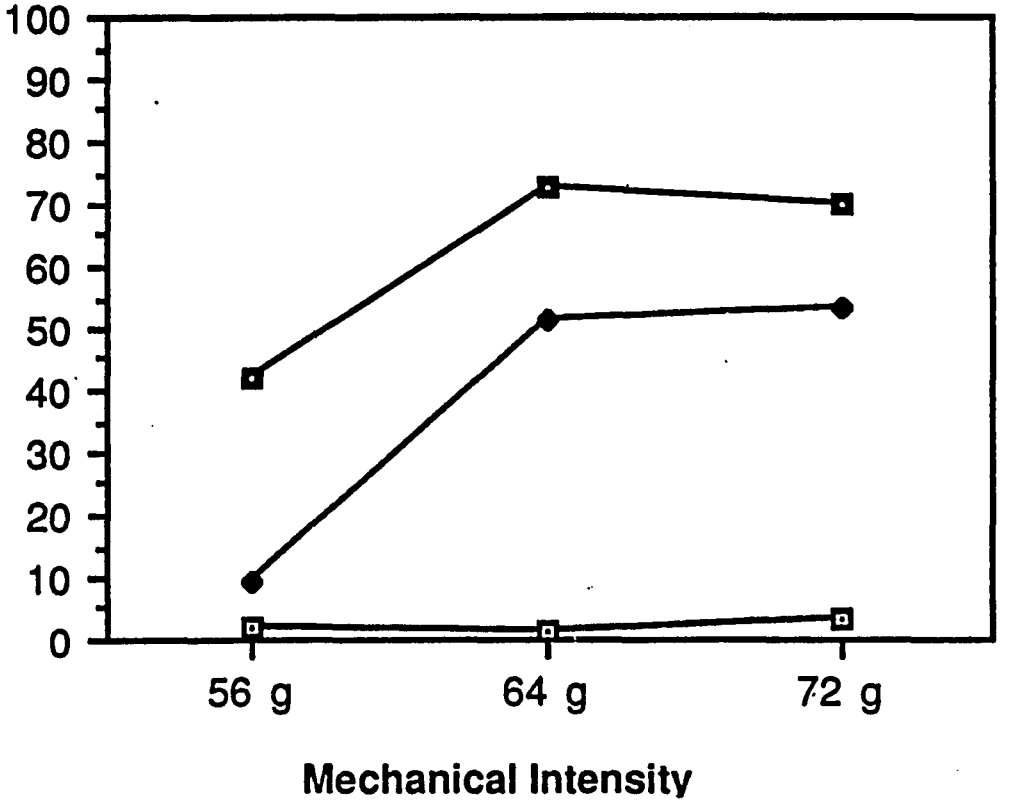

Forepaw
Hindpaw
Tail
Response Latency (sec.)
5
4
3
2
1
● = Thermal
○ = Mechanical
0 5 10 20 0 5 10 20 0 5 10 20
Dose (μg)

Figure 7. Withdrawal response latencies expressed in M.P.E. as a function of thermal and mechanical intensity in 3 day old rats intrathecally injected with NE. Each value represents the mean withdrawal response latency of six rats. S.E.M.s for all means were less than ±0.30. Details of dosages and injection procedures are described in the text.

3 Day Olds


100
90
80
70
60
50
40
30
20
10
0
forepaw
hindpaw
tail
42°C
47°C
52°C
Thermal Intensity
Response Latency (MPE)


100
90
80
70
60
50
40
30
20
10
0
56 g
64 g
72 g
Mechanical Intensity

Discussion

Experiment 1 indicates that intrathecal injections of NE and clonidine produced modest but significant analgesia in the preweanling rat. This response was more pronounced and developed earlier when the pain inducing stimulus was mechanical rather than thermal. This finding is consistent with those of investigators whose work is cited above. It must be noted that the comparison of response latency and topography to stimuli representing different pain modalities is limited by the problem of equating stimulus intensity. In order to analyze the "thermal vs mechanical" aspects of this study, the stimulus intensities of the two testing procedures must be equated. Matching the discharge frequencies in cell populations presumed to subserve thermo- and mechano-nociception may represent the most precise means of achieving this goal. A behavioral alternative to electrophysiology is to operationally define "equated stimulus intensity" in terms of the animal's response. Manipulating stimulus intensities until baseline response (appendage withdrawal) latencies for thermal and mechanical tests are matched is one means of approach. However, in the drug studies described above, we found that exactly matching mechanical and thermal latencies required the use of weights (56 grams) that produced ambiguous responses, or temperatures (54^{o}C) that potentially produced tissue damage. Therefore, the thermal and mechanical stimulus intensities of 47^{o}C and 64 grams used in this study represented the lowest intensities that produced a consistent withdrawal response. The differences in the data of control animals that reflected differences in baseline responding between thermal and mechanical tests were not significant.

It could be argued that the magnitude of stimulus intensity itself determines whether or not a noradrenergic receptor system becomes instrumental in pain control. Differences between thermal and mechanical response curves observed in NE-treated

rats at different ages might be due to a simple mismatch in intensity between the two stimuli rather than to a direct action of the drug. If this were true, then water temperature could be manipulated until the thermal response curve for 3 day old rats, for instance (see Figure 4), approximated the mechanical response curve. The results of the intensity function study in NE-treated, 3 day olds suggest that noradrenergic receptor mediation of pain control is not recruited simply when intensity levels reach a certain value (see Figure 7). Over a range of thermal and mechanical stimulus intensities, the respective response curves never resembled each other except for the forepaw which was unresponsive to any dose of intrathecal NE at 3 days of age for either sense modality. The increase in tail withdrawal latency observed at low intensity levels does not lend itself to either a stimulus intensity or drug action model and remains unexplainable.

In addition to a possible mismatch in stimulus intensities between the two algesic tests within a subject, it must be noted that the thermal and mechanical intensities of 47^{o}C and 64 grams respectively were used during drug testing irrespective of the animal's age. The assumption that a 64 gram weight applied to the tail of 3 and 10 day old rats represents a like experience with respect to intensity is of course without standing. Even a cursory observation of rat pup appendages reveals a pronounced difference in size, thickness of skin, amount of fur, motor agility and degree of spontaneous movement between the two ages. These ontogenetic differences undoubtedly translate into intensity differences when the stimulus magnitude is held constant over different ages during algesic testing. However, preliminary attempts to adjust stimulus intensity to subject age resulted in withdrawal responses that were not consistent in latency, duration or topography. Again, the thermal and mechanical stimulus intensities of 47^{o}C and 64 grams used in these drug studies represented the lowest intensities that yielded unambiguous withdrawal responses across all ages tested. This

inability to match stimulus intensity with subject age for practical test purposes reflects a presently unavoidable crudeness in the behavioral assays used to measure analgesia in this study. Whether or not algesic tests sensitive enough to accommodate age differences in neonatal animals can feasibly be implemented remains to be determined. One obstacle to the design of such tests is the fact that development is not a uniform process that permits all factors influencing pain responses to be accounted for by changes in one stimulus parameter, i.e. intensity. Do ontogenetic changes in the degree of vascularization, skin thickness, muscular growth, nociceptor distribution, etc. occur at uniform rates such that stimulus intensity can be adjusted in a precise enough manner to result in equated response latencies across ages? The answer to this question requires further investigation. However, results of the variable intensity study in NE-treated rats described in the preceding paragraph demonstrate that stimulus intensity in and of itself does not determine whether or not spinal noradrenergic systems become active in regulating pain signals. Thus, age differences in the spinal effects of exogenously applied NE and noradrenergic agonists found in these studies are not simply the result of a standardized stimulus being more intense for younger than for older animals. The test procedures described in this thesis provide a tentative behavioral model for studying the development of pain responses in preweanling rats. It should be noted that the variable intensity study described above represents a preliminary investigation, and the extent to which NE mediated analgesia is differentially influenced by mild vs intense stimuli within any one pain modality requires further examination. However, the finding that NE induced analgesia was more pronounced against a mechanical stimulus when both opioid (Takagi, Satoh, Akaike, Shibata and Kuraishi, 1977; Kuraishi, Satoh, Harada, Akaike, Shibata and Takagi, 1980) and non-opioid systems were challenged suggests that the relationship between different sense modalities can be operationally quantified.

Another concern with behavioral assays that measure pain responses is the degree to which response magnitude is graded in proportion to stimulus intensity (Vierck and Cooper, 1984). Our finding that little responding occurred in nontreated rat pups at water temperatures less than 40°C indicated that the sensation of "wetness" was not a stimulus quality capable of evoking withdrawal behavior. More importantly, response latencies continued to gradually decline as water temperature increased above 42°C. The gentle slope of the response curve beyond the temperature that presumably resulted in nociception would be predicted by limitations in the velocity of reflexive responding. A point is reached where the animal simply cannot withdraw any faster despite the intensity of the stimulus. Although other properties of the response were not quantified, observation suggested that duration of the withdrawal behavior decreased and response force increased as the stimulus intensified. Also, the frequency of vocalization and squirming appeared to increase with higher water temperatures. However, the extent to which these more highly organized behaviors reflected changes in general arousal levels independent of pain sensation could not be readily determined.

The increase in response latency produced by intrathecal clonidine against a mechanical stimulus appeared to occur most prominently at about 10 days of age, especially for the tail. This finding is consistent with that of Simmons and Jones (1985) who found that NE-stimulated cyclic AMP accumulation in whole rat spinal cord peaked at age 12 days and declined to adult levels at age 30 days. It is possible that the shorter thermal response latencies that occurred at 14 days in the current study are reflective of the decline in NE-stimulated cyclic AMP observed from age 12 to 30 days. Regionally, however, such accumulation peaked at 15 days in lumbar cord. This observation would predict a stronger analgesic response in the tail at 14 days than was demonstrated. An alternative explanation for the reduced response latencies at this age is that the relatively pronounced growth of the rat spinal cord between 10

and 14 days necessitated a greater drug dose and/or volume to reach all the cord sites that were accessed at 10 days of age. As of the present time, virtually nothing is known about the in vivo development of adrenergic receptors or how they functionally contribute to behavior in maturing organisms. Although accumulation of NE-stimulated cyclic AMP indicates the degree of sensitivity of adrenergic receptors to the action of NE, it must be noted that NE storage and uptake mechanisms in brain are present at birth in activity levels comparable with adults (Coyle and Molliver, 1977), while NE innervation does not reach maximum density until 40 to 60 days of age (Coyle,1974). Therefore, inferences drawn from the correlation of changes in a specified neural substrate with changes in a class of behaviors must be interpreted cautiously.

It must be mentioned that although intrathecal clonidine produced a significant thermal analgesic effect, the highest dose at 10 days of age resulted in mean response latencies of no greater than about 2 seconds. The mean baseline latency for all limbs at age 10 days was .73 seconds (N=75). Although the time difference between .73 and 2 seconds is small, this baseline-test difference is comparable with that found in previous adult studies that employed a thermally noxious stimulus and that were deemed to be analgesic (Reddy et al., 1980). Also, the analgesic effects of adrenergic compounds do not appear to be secondary to local vasoconstriction in the spinal cord. Epinephrine applied directly to the brain resulted in no constriction of arterioles and only slight constriction of larger arteries (Florey, 1925; Fog, 1939; Wei, Raper, Kontos and Patterson, 1975). Serotonin is a more potent vasoconstrictor of cerebral vessels than NE (Bohr, Goulti and Taquini, 1961; Toda and Fujita, 1973) yet was much less potent than NE in producing analgesia when applied intrathecally (Wang, 1977; Yaksh and Wilson, 1979). In addition, the potent vasoconstrictor angiotensin-II did not produce analgesia when intraspinally administered (Wei, Kontos and Patterson,

1978). The alteration of pain thresholds by intrathecal clonidine or NE did not therefore appear to be the result of local cord ischemia.

The anatomic distribution of noradrenergic receptors with respect to α_1- and α_2-NE subtypes does not appear to be uniform in the spinal dorsal horn of the adult. The terminals of small, unmyelinated afferents were found to be located primarily in layers I and II, a region that demonstrated a high density of α_2- but not α_1-NE receptor sites (Dashwood et al., 1985; Young and Kuhar, 1980). That a pain related behavior is organized around a specified receptor system through all stages of development is an assumption that should not remain unchallenged. Neural substrates presumed to regulate specified behaviors frequently recruit or discard functional mechanisms in the process of growth (Turkewitz and Kenny, 1982). As mentioned above, Hamm and Knisely (1984) demonstrated that shock induced analgesia was mediated by an opioid system in the 28 day old rat that yielded to a non-opioid system as the rat matured. However, the failure of intrathecal phenylephrine to alter nociceptive thresholds in our study suggests that the minor role of the spinal noradrenergic α_1 receptor in adult pain regulation is afforded no greater importance in the infant rat.

The preferential expression of the mechanical analgesic effect in the hindpaw and tail is consistent with related studies indicating developmental differences in the ability of opiates to produce analgesia in the paws compared to the tail. For instance, the analgesic effects of ketocyclazocine preceded morphine's analgesic effects (Barr et al., 1986) against both thermal and mechanical stimuli in the tail by several days. Ketocyclazocine produced analgesia between 7 and 10 days of age, while the effects of morphine did not peak until day 14. In the forepaw test, morphine was more effective than ketocyclazocine with both thermal and mechanical stimuli (Giordano and Barr,

1987). Furthermore, opioid receptor mediated analgesia is also influenced by the type and intensity of the noxious stimulus (Dennis, Melzack, Gutman and Boucher, 1980; Cannon, Terman, Lewis and Liebeskind, 1984). The extent to which noradrenergic receptor mediated analgesia develops differentially with respect to body area remains to be determined. The current study indicates a possible caudal to rostral emergence of NE related analgesia against a mechanical stimulus. However, the intrathecal injection procedure precluded confinement of the drug to specified regions of the spinal axis (cervical, thoracic, lumbar) or to specified laminae within the cord (dorsal vs ventral horn). Consequently, different populations of afferents known to terminate in separate layers of the spinal dorsal horn or along different segments of the spinal axis could not be selectively exposed to drug by this procedure. Limb effects were interpreted from data that represented total or near total exposure of spinal cord to drug compounds. The degree to which opioid and non-opioid mediated analgesia develop differentially as a function of stimulus intensity, though outside the scope of this study, awaits investigation.

Finally, it must be mentioned that although subjects demonstrating supraspinal staining following the dye verification procedure were excluded from the data, it is likely that some of the intrathecally applied drug redistributed to brain sites in subjects that did not display such staining. The lipid partition coefficients of heavy dyes such as cresyl violet are likely to differ significantly from those of lipophilic compounds such as NE. Also, Kupferberg and Way (1963) observed that morphine gains easier access to the brain in young rats than in adults. Therefore, in spite of the findings in adult animals mentioned previously (Yaksh and Rudy, 1976; LoPachin and Rudy, 1982; Schmauss et al., 1983) predicting the spread of NE along the spinal axis of young rats based on the distribution of intrathecally injected cresyl violet dye can only be done in a tentative way. However, three observations indicate that the findings

in this study resulted from the action of drug restricted to the spinal cord: a) The relatively short time period between injection and testing (15 min.) minimized the redistribution of drug from spinal to supraspinal sites. b) A large dose of clonidine injected into the jugular vein produced no analgesic effects. c) Previous studies indicate that NE applied to brain sites produces hyperalgesia rather than analgesia (see Experiment 2, Spinal Opioid-Noradrenergic Interactions).

Experiment 2

Spinal Opioid-Noradrenergic Interactions

In general, opioid links in pain modulatory systems appear to be prevalent in the brainstem, RVM, and spinal dorsal horn. The following describes relevant anatomical and behavioral studies for each of these three areas.

In the brainstem, abundant opiate receptors (Atweh and Kuhar, 1977b) and enkephalin and dynorphin immunoreactive perikarya were found in PAG (Hokfelt, Ljungdahl, Terenius, Elde and Nilsson, 1977; Uhl, Goodman, Kuhar, Childers and Snyder, 1979; Finley, Maderdrut and Petrusz, 1981; Watson, Khachaturian, Akil, Coy and Goldstein, 1982; Moss, Glazer and Basbaum, 1983). The morphine antagonist naloxone injected into this area reversed analgesia induced by systemic morphine (Tsou and Jang, 1964; Yeung and Rudy, 1980). Clinical studies indicate that electrical stimulation of PAG resulted in verbal reports of pain relief (Hosobuchi, Adams and Linchitz, 1977; Mazars, Merienne and Ciolola, 1979). These findings underscore the importance of PAG in modulating opioid induced analgesia in addition to its role in non-opioid control of pain activity.

Several RVM nuclei that contribute descending fibers to the dorsal horn via the DLF were responsive to morphine injection. Nanogram doses of morphine applied to the nucleus reticularis paragigantocellularis (Takagi, Doi and Akaike, 1976; Akaike et al., 1978) resulted in analgesia that was blocked by systemic naloxone (Satoh et al., 1980) and intrathecal phenoxybenzamine, an α-noradrenergic antagonist (Kuraishi, Harada, Satoh and Takagi, 1979a). Enkephalin containing neurons have been located in the nucleus reticularis gigantocellularis pars alpha (Hokfelt et al., 1979) and in the more medially located NRM (Beitz, 1982). The latter nucleus was also sensitive to opiate

microinjections (Fields and Anderson, 1978; Dickenson et al., 1979; Lebars, Dickenson and Besson, 1980). Electrostimulation of these RVM nuclei resulted in behavioral analgesia that was blocked by intrathecal naloxone (Zorman, Belcher, Adams and Fields, 1982). However, it is not clear from these findings whether or not cells from RVM nuclei release opioids directly to the spinal cord. The ability of intrathecal naloxone to reverse analgesia induced by RVM stimulation may be due to the action of local opioid containing interneurons in the cord.

In the dorsal horn, immunocytochemical studies have established the presence of enkephalin (Glazer and Basbaum, 1981; Hunt, Kelly, Emson, Kimmel, Miller and Wu, 1981; Cruz and Basbaum, 1985) and dynorphin (Cruz and Basbaum, 1985) in layers I, II, and V. This area was also found to be rich in opioid receptors (LaMotte, Pert and Snyder, 1976; Atweh and Kuhar, 1977a; Herkenham and Pert, 1980). Enkephalin synapses on dorsal horn afferents have been characterized by Ruda (1982) and although enkephalin levels were unaffected by dorsal rhizotomy (Hokfelt et al., 1977) the number of opioid receptors was diminished significantly (LaMotte et al., 1976). These observations suggest that while dorsal horn enkephalin derives from intrinsic spinal interneurons, opioid receptors exist on primary afferent terminals. That these studies may be relevant to antinociception was evidenced by the analgesia that resulted when opiate agonists were injected into the spinal cord (Yaksh and Rudy, 1976) and by the selective blockade of second order afferents by enkephalin iontophorized in the dorsal horn (Duggan, Hall and Headley, 1977).

Morphine injected at spinal and supraspinal sites concurrently resulted in more pronounced analgesia than at either site alone (Yeung and Rudy, 1980). This enhanced action was observed previously when dose response curves indicating the effects of systemically applied morphine on thermal tests were shifted to the right by naloxone applied intracerebrally or spinally (Yaksh and Rudy, 1978). Because descending

noradrenergic cells mediate some of the effects of morphine applied to brainstem sites (Hammond, 1986), it is likely that noradrenergic compounds and morphine synergize to produce analgesia when both are administered spinally (see below). Analgesia might then be demonstrated with combined doses of these compounds that alone would effect no change in pain thresholds (Yaksh, 1986).

The contribution of opioid peptides and NE to the antinociceptive effects produced by each other is complicated. As indicated in a previous section of this thesis, the nuclei from which noradrenergic cells originate are diffusely located. Interestingly, Kuhar (1981) found much overlap in the distribution of α_2 and μ-opioid binding sites in the central nervous system. Yaksh (1986) reported that opiates and α_2 agonists appear to affect dorsal root ganglion cells, dorsal horn neurons, and primary afferent excitability in comparable ways. Also, recent intrathecal studies indicated a significant leftward shift in the dose response for morphine when coadministered with α_2 agonists such as NE, clonidine and ST-91 (Wang, Yasuoka and Yaksh, 1980; Yaksh and Reddy, 1981; Hylden and Wilcox, 1983). Whether or not this effect was produced by the release of opioid peptides that interacted directly with noradrenergic cells in the dorsal horn is not clear. Analgesia induced by spinally applied NE was found to be naloxone reversible and to be cross tolerant with morphine's analgesic effects in some intrathecal studies (Loomis, Jhamandas, Milne and Cervenko, 1987) but not others (Reddy and Yaksh, 1980). Although the activation of opioid and adrenergic receptors in spinally mediated antinociception seems likely, a neuromodulatory role for the endogenous substances that interact with these receptors cannot be ruled out. Both NE and serotonin have been shown to inhibit the in vitro catabolism of Met-enkephalin in brain tissue (Jakubovic, 1982). Evidence suggests that although an opioid-NE link appears likely in supraspinal sites, noradrenergic and enkephalinergic inputs to the dorsal horn are capable of modulating pain signals independently of each other. Phentolamine intrathecally

introduced decreased analgesia produced by PAG morphine microinjection (Yaksh, 1979), systemic morphine (Proudfit and Hammond, 1981), intrathecal NE (Reddy et al., 1980), but not intrathecal morphine (Russell and Yaksh, 1981). Also, whereas autoradiographic and immunocytochemical studies have revealed a serotonin-enkephalin connection in the dorsal horn (Glazer, Steinbusch, Verhofstad and Basbaum, 1981; Basbaum, Glazer and Lord, 1982), no such link has been established for NE and enkephalin.

In addition, NE appears to exert antinociceptive effects independent of interneurons containing major non-opioid, inhibitory transmitters in the spinal cord. Analgesia resulting from intrathecal NE was not antagonized by picrotoxin, a GABA antagonist (Johnston, 1976 for review; Reddy et al., 1980) or strychnine, a glycine antagonist (Curtis, Duggan and Johnston, 1968; Reddy et al., 1980). Yet, these two transmitters are present in as many as 50% of all synaptic terminals in the spinal cord (Iverson and Bloom, 1972).

Whereas NE activity in the spinal cord serves to promote analgesia, several studies have demonstrated that supraspinal NE activity results in the lowering of pain thresholds. NE injected intraventricularly reversed analgesia induced by systemic morphine (Sparkes and Spencer, 1971; Calcutt, Hangley, Sparkes and Spencer, 1973). Conversely, analgesia produced by PAG stimulation was enhanced following the administration of disulfiram, a dopamine-β-hydroxylase inhibitor (Akil and Liebeskind, 1975). Also, various noradrenergic antagonists were found to promote analgesia when applied to RVM nuclei (Hammond, Levy and Proudfit, 1980; Sagen and Proudfit, 1985). Cicero, Meyer and Smithloff (1974) have demonstrated that the noradrenergic receptor blockers phentolamine and phenoxybenzamine injected intraperitoneally resulted in analgesia. These same antagonists also enhanced analgesia induced by morphine.

These results support the suggestion by Basbaum, Moss and Glazer (1983) that in the RVM, NE modulates pain information by tonically inhibiting descending serotonergic cells in the nucleus raphe magnus. Furthermore, this noradrenergic input may be presynaptically controlled by enkephalinergic interneurons. In fact, Llorens, Martres and Baudry (1978) have found that opiates bind to terminals of noradrenergic neurons in the cerebral cortex. As the electrophysiological effects of opioids are usually inhibitory (North, 1979; Nicoll, Alger and Nicoll, 1980), any area in which electrical stimulation and morphine injection elicit a like excitatory response indicates a disinhibitory role for the opioid cells in question. In fact, the net opiate response in supraspinal sites appears to be excitatory (Fields, 1984). Such a circuitry could account for the previously mentioned observation that both electrical stimulation and morphine microinjection in the nucleus raphe magnus produced analgesia.

Aim of Study

This study investigated the characteristics of opioid-noradrenergic interactions in modulating pain responses in the developing rat. More specifically, the contribution of spinal noradrenergic input to morphine induced analgesia was addressed. As mentioned above, intrathecal injections in the adult rat have demonstrated a leftward shift in the opioid dose response curves for coadministered morphine and α_2 agonists (Wang et al., 1980; Yaksh and Reddy, 1981; Hylden and Wilcox, 1983). That descending noradrenergic cells mediate some of the effects of morphine applied to brain sites has been shown by other studies cited above (Kuraishi et al., 1979a; Yaksh, 1979; Proudfit and Hammond, 1981). Still other investigators have demonstrated that at the spinal level, morphine is capable of producing analgesia independently of noradrenergic input (Reddy and Yaksh, 1980; Russell and Yaksh, 1981). These studies indicate that in the

adult rat, noradrenergic control of opioid mediated analgesia occurs at the supraspinal but not spinal level. The following experiments investigated the ability of a spinally applied α-noradrenergic antagonist to alter the analgesic effects of morphine applied directly to either the brain or spinal cord of neonatal rats. Whether analgesia produced by the stimulation of opiate receptors in the brain is mediated by centrifugal noradrenergic activity or by the activation of noradrenergic terminals at the spinal level would be investigated in infant rats. Morphine was applied to the brain by free-hand, intracerebroventricular injection as described in the methods section below or was applied to the spinal cord by intrathecal injection as described in Experiment 1 with some modifications as indicated below. Although intrathecal phentolamine has been shown to reverse the analgesic effects of morphine applied to the brain in adult animals, the functional ontogeny of this noradrenergic-opioid interaction has not yet been demonstrated. Also, if intrathecal phentolamine can be shown to block analgesia produced by intrathecal morphine, a spinal noradrenergic-opioid link would be indicated in young rats that is presumed to modulate pain in adult animals only at supraspinal sites. A developmental shift in the functional mechanisms underlying pain control would thus be demonstrated. Although yohimbine is more selective for the noradrenergic α_2 receptor than is phentolamine, the latter compound was used in this investigation in order to be consistent with previous intrathecal studies, many of which used phentolamine to block the analgesic effects of noradrenergic agonists.

Materials and Methods

Subjects. Neonatal rats described in the previous experiment were also used in this study. Three pups per litter were used for each of three morphine treatment groups. Within each morphine group, littermates received one of three doses of intrathecal phentolamine. Therefore, nine pups from three separate litters represented one complete treatment group consisting of either 3 or 10 day old rats.

Injection. All subjects were surgically implanted with intrathecal catheters exactly as described in Experiment 1. Twenty-four hours later, pups were injected intrathecally with phentolamine in 4 µl solution. Immediately following, morphine was administered directly to the brain by free-hand, intracerebroventricular injection as described by Klee and Praestholm (1976) with modifications for infant rats. For this procedure, a 30 gauge hypodermic needle attached to a 10 µl Hamilton syringe containing a 1 µl solution of morphine and cresyl violet dye was prepared. The subject's head was shaved and moistened with a drop of water to permit visualization of bregma and the midline suture. The unanesthetized, hand-held pup was bound lightly in cloth with head exposed during penetration of the cartilaginous skull by the needle. The morphine solution was injected free-hand into the left ventricle of the brain over a period of 5 seconds. Another 3 seconds elapsed before withdrawing the needle to insure that all of the drug was absorbed into the ventricle. Placement of the needle into the left ventricle was mapped according to a stereotaxic atlas of the developing rat brain (Sherwood and Timiras, 1970). The coordinates used for each age group of rats differed. For ten day olds, the left ventricle was determined to be .9 mm rostral from bregma, 1.4 mm lateral from 0 (the midline suture), and 3.8 mm dorsal to ventral. The depth of penetration was guided by a precut needle collar whose length was determined by the age of the animal. The collar fit firmly over the needle thus exposing a length of shaft equal to the dorsal to ventral coordinate for each age group. Fifteen minutes following injection, all pups

were tested for analgesia in the manner described in Experiment 1 under "Drug Tests". Each animal was tested thermally and mechanically.

Following testing, the verification procedure for intrathecal injection described in Experiment 1 was performed to insure that the spread of dye along the spinal axis was sufficient. To verify correct placement of the ventricle injection, carcasses were placed in deep freeze. Pups were then decapitated and the skin above the injection site removed. A small contusion clearly marked the needle penetration site. The head was sliced coronally through the contusion and permitted to partially thaw. Visual examination of the tissue revealed cresyl violet staining throughout the ventricular area including the third ventricle in those subjects successfully injected. Litters containing pups that did not display such staining (about 30%) were excluded from the data.

Minimizing the total fluid volume injected into the subdural space of neonatal rats receiving both intrathecal morphine and phentolamine was achieved in the following manner. A 10 µl Hamilton syringe was filled with 4 µl of phentolamine solution and inserted .25 cm into the exposed end of the catheter. One µl of solution was injected into the animal leaving 3 µl solution in the catheter. Three µl saline was then slowly injected through the catheter to clear the remaining 3 µl of drug into the animal. Two cm of the catheter tip was cut off. Five minutes later, 4 µl morphine was injected into the subdural space. A speck of cyano acrylate sealed the exposed end of the silastic to prevent the remaining 1.5 µl of drug from seeping into the cord. Ten minutes following injection, all pups were tested for analgesia as described in Experiment 1 under "Drug Tests".

Drugs. All drugs for intrathecal and intracerebroventricular injections were freshly prepared in physiologic saline. The following drugs were used: Morphine sulphate and phentolamine HCl (Sigma Chemical Co., St. Louis, Mo.).

Statistics. Litters were treated as experimental units (Denenberg, 1977). The dose of morphine, the dose of phentolamine, limb, stimulus and age effects were compared by means of a five-way factorial analysis of variance for subjects receiving intraventricular morphine. No age effects were compared for subjects receiving intrathecal morphine as only one age group was tested. Repeated measures were done for stimulus, limb and dose phentolamine as each animal received both test stimuli and all appendages tested were from the same animal. All doses of phentolamine were administered to pups from the same litter. For a given interaction whose overall effects were found to be significant, Scheffe's S method (Kirk, 1968) was used as a post hoc test to determine whether individual means were significantly different from each other.

Results

Intraventricular Morphine and Intrathecal Phentolamine. Intraventricular injections of morphine produced a dose-dependent increase in response latency on both thermal and mechanical tests. Figure 8 represents withdrawal response latency as a function of dose morphine for each of three doses of phentolamine in 3 day old rats. The analgesic effect of 30 μg morphine was antagonized by intrathecal phentolamine when a mechanically noxious stimulus was employed. The latency to respond was significantly less in pups that received 30 μg phentolamine than in those that received 15 μg phentolamine or vehicle only [dose phentolamine X dose morphine; $F, (4, 30) = 5.59, p < .01$]. Post hoc analysis of the test means indicated that phentolamine had no effect on latencies increased by morphine when a thermally noxious was used. The differences in response latencies that appear with saline vehicle in both thermal and mechanical tests were not significant. Six litters per age group were used with a roughly equal distribution of males and females per litter. Table 1 displays the mean ± SEM for each value plotted in Figure 8.

Figure 9 represents withdrawal response latency as a function of dose morphine for each of three doses of phentolamine in 10 day old rats as for the 3 day olds. The analgesic effect of 30 μg morphine was antagonized by intrathecal phentolamine when a mechanically noxious stimulus was used. The latency to respond was significantly less in phentolamine treated pups than in those that received only saline [dose phentolamine X dose morphine; $F, (4, 30) = 5.09, p < .01$]. Phentolamine failed to antagonize significantly the analgesic effects of morphine at 30 μg against a thermally noxious stimulus. Significant limb effects were found only by collapsing data across two or more variables. In any interaction combining stimulus and morphine effects, no significant limb effects were found in either 3 day olds [stimulus X limb X dose morphine; $F, (4, 30) = 2.49, p > .05$] or 10 day olds [stimulus X limb X dose morphine; $F, (4, 30) =$

Figure 8. Effects of intraventricular morphine on thermal and mechanical response latencies in 3 day old rats pretreated with intrathecal phentolamine. Each value represents the mean withdrawal response latency of six rats. S.E.M.s for all means were less than ±0.55. Detail of dosages and injection procedures are described in the text.

3 Day Olds

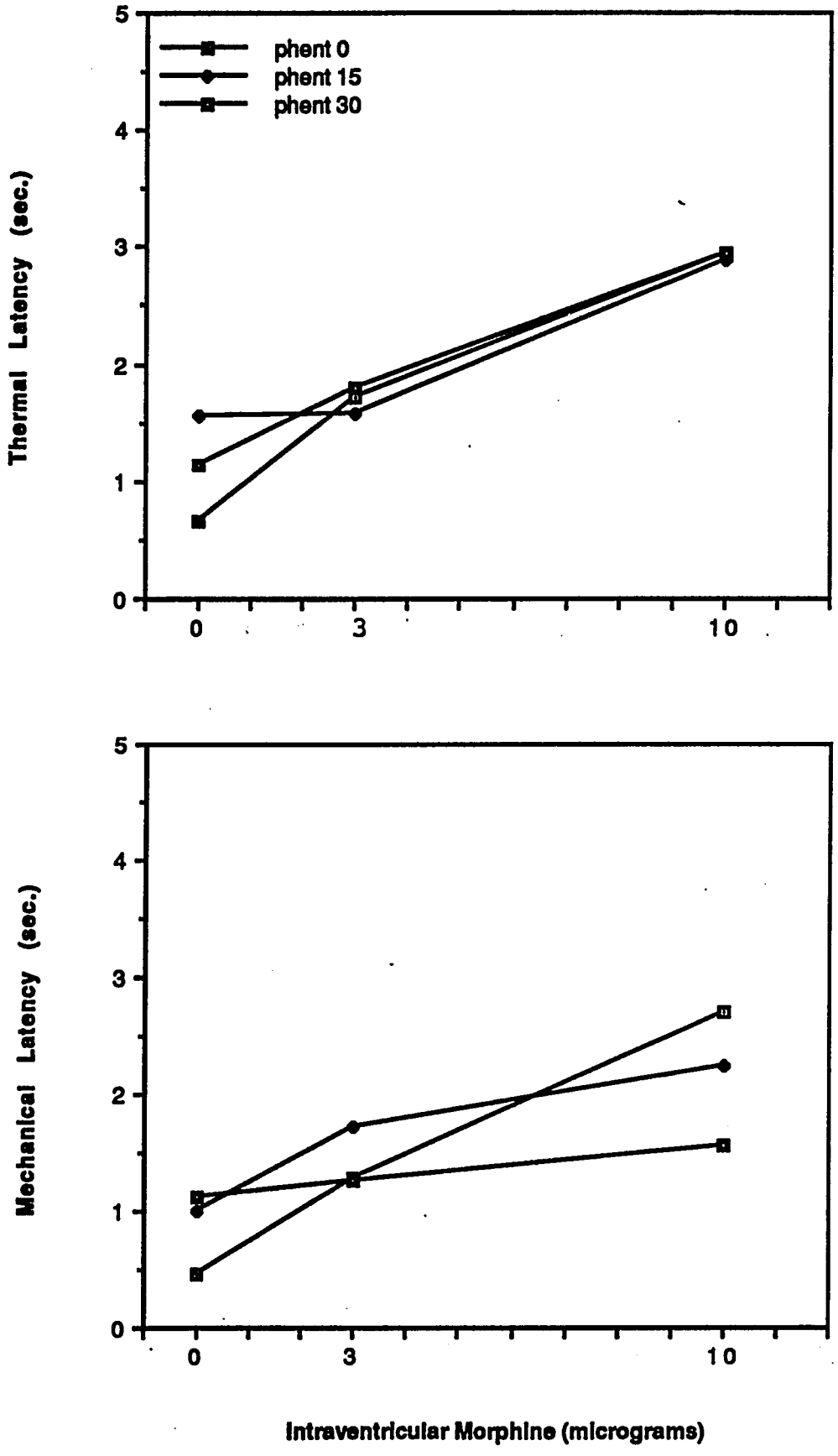

phent 0
phent 15
phent 30
Thermal Latency (sec.)
Mechanical Latency (sec.)
0
1
2
3
4
5
0
3
10
Intraventricular Morphine (micrograms)

Table 1

Mean and Standard Error Mean for Each Value Plotted in Figure 8

Dose ICV Morphine (µg) for Thermal Test

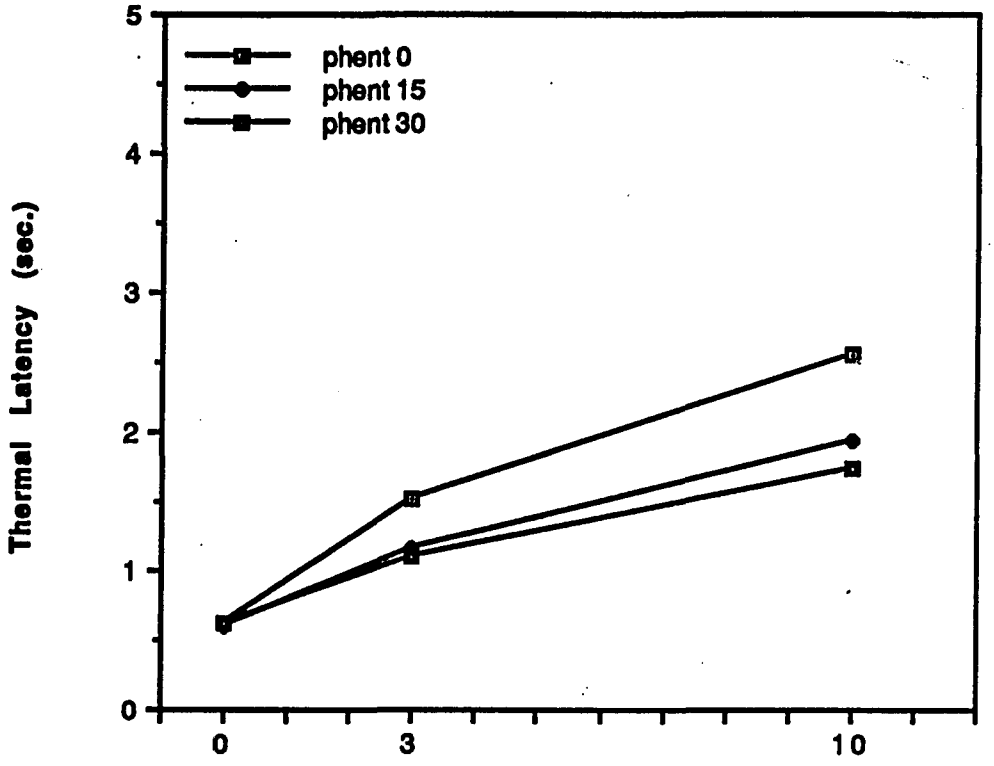
Dose IT Phentolamine (µg)	0	3	10
0	.66 ± .05	1.71 ± .13	2.95 ± .18
15	1.56 ± .33	1.58 ± .33	2.88 ± .32
30	1.13 ± .24	1.80 ± .54	2.95 ± .29

Dose ICV Morphine (µg) for Mechanical Test

Dose IT Phentolamine (µg)	0	3	10
0	.47 ± .04	1.29 ± .18	2.69 ± .19
15	1.01 ± .23	1.72 ± .23	2.25 ± .33
30	1.13 ± .39	1.27 ± .43	1.56 ± .25

Figure 9. Effects of intraventricular morphine on thermal and mechanical response latencies in 10 day old rats pretreated with intrathecal phentolamine. Each value represents the mean withdrawal response latency of six rats. S.E.M.s for all means were less than ± .44. Detail of dosages and injection procedures are described in the text.

10 Day Olds


phent 0
phent 15
phent 30
Thermal Latency (sec.)
0
1
2
3
4
5
0
3
10

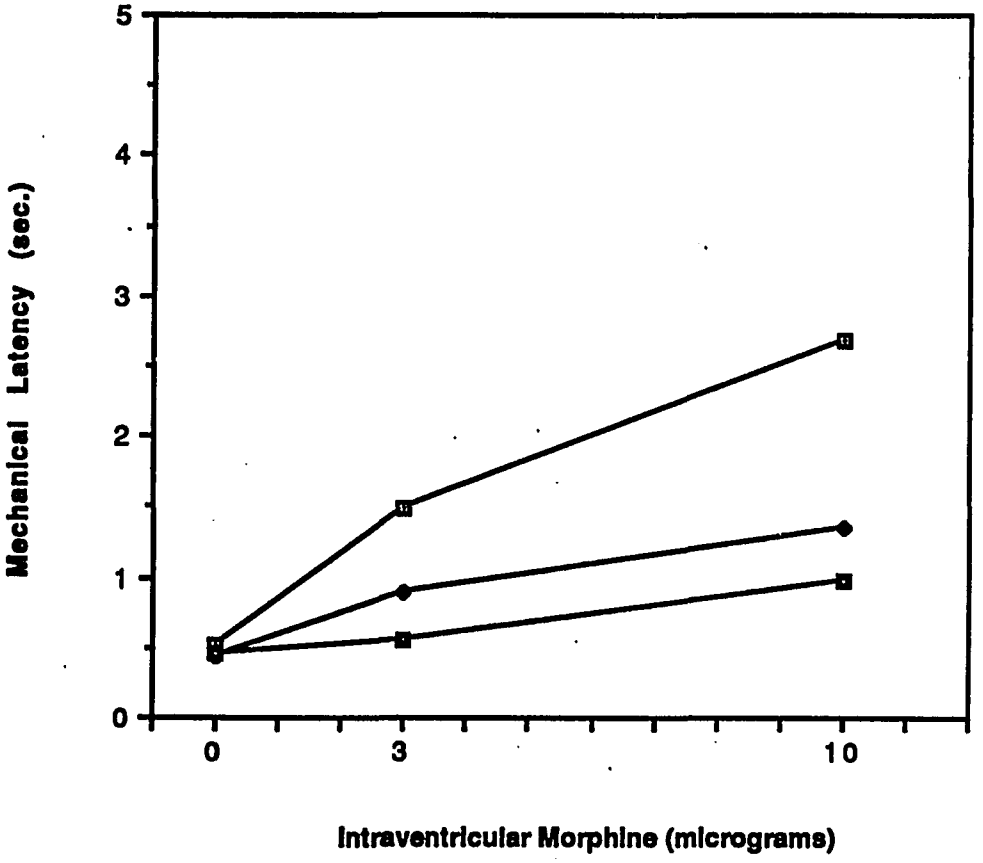

Mechanical Latency (sec.)
0
1
2
3
4
5
0
3
10
Intraventricular Morphine (micrograms)

Table 2

Mean and Standard Error Mean for Each Value Plotted in Figure 9

Dose IT Phentolamine (μg)	Dose ICV Morphine (μg) for Thermal Test		
	0	**3**	**10**
0	.63 ± .05	1.52 ± .21	2.56 ± .34
15	.60 ± .06	1.16 ± .31	1.94 ± .40
30	.62 ± .07	1.09 ± .23	1.74 ± .43

Dose IT Phentolamine (μg)	Dose ICV Morphine (μg) for Mechanical Test		
	0	**3**	**10**
0	.51 ± .05	1.49 ± .23	2.67 ± .22
15	.44 ± .04	.90 ± .20	1.35 ± .24
30	.45 ± .05	.55 ± .07	.97 ± .18

.75, $p > .05$]. Six litters per age group were used with a roughly equal distribution of males and females per litter. Table 2 displays the mean ± SEM for each value plotted in Figure 9.

Intrathecal Morphine and Intrathecal Phentolamine. Figure 10 represents withdrawal response latency as a function of dose morphine for each of three doses of phentolamine in 10 day old rats. Intrathecal injections of morphine produced a dose-dependent increase in response latency on both thermal and mechanical tests [$F, (2, 15) = 38.45$, $p < .01$]. Intrathecal phentolamine did not alter significantly the response latencies elevated by any dose of spinally injected morphine against either stimulus type [$F, (4, 30) = .39$, $p > .05$]. Six litters were used with a roughly equal distribution of males and females per litter. Table 3 displays the mean ± SEM for each value plotted in Figure 10.

During intraventricular morphine testing, only 2 animals ever reached the cut-off latency of 5 sec. for either age and either sense modality. This occurred with the highest dose of morphine only (10 μg). Tests using intrathecal morphine resulted in a slightly higher percentage of animals reaching the cut-off latency, but again, only with the highest dose of morphine (30 μg) and regardless of the dose of intrathecal phentolamine. This finding provides further evidence that morphine did not simply inactivate the animal so that responding could not occur irrespective of sensation. The use of latencies rather than MPEs to represent these data is therefore justified.

Figure 10. Effects of intrathecal morphine on thermal and mechanical response latencies in 10 day old rats pretreated with intrathecal phentolamine. Each value represents the mean withdrawal response latency of six rats. S.E.M.s for all means were less than ± .79. Detail of dosages and injection procedures are described in the text.

10 Day Olds

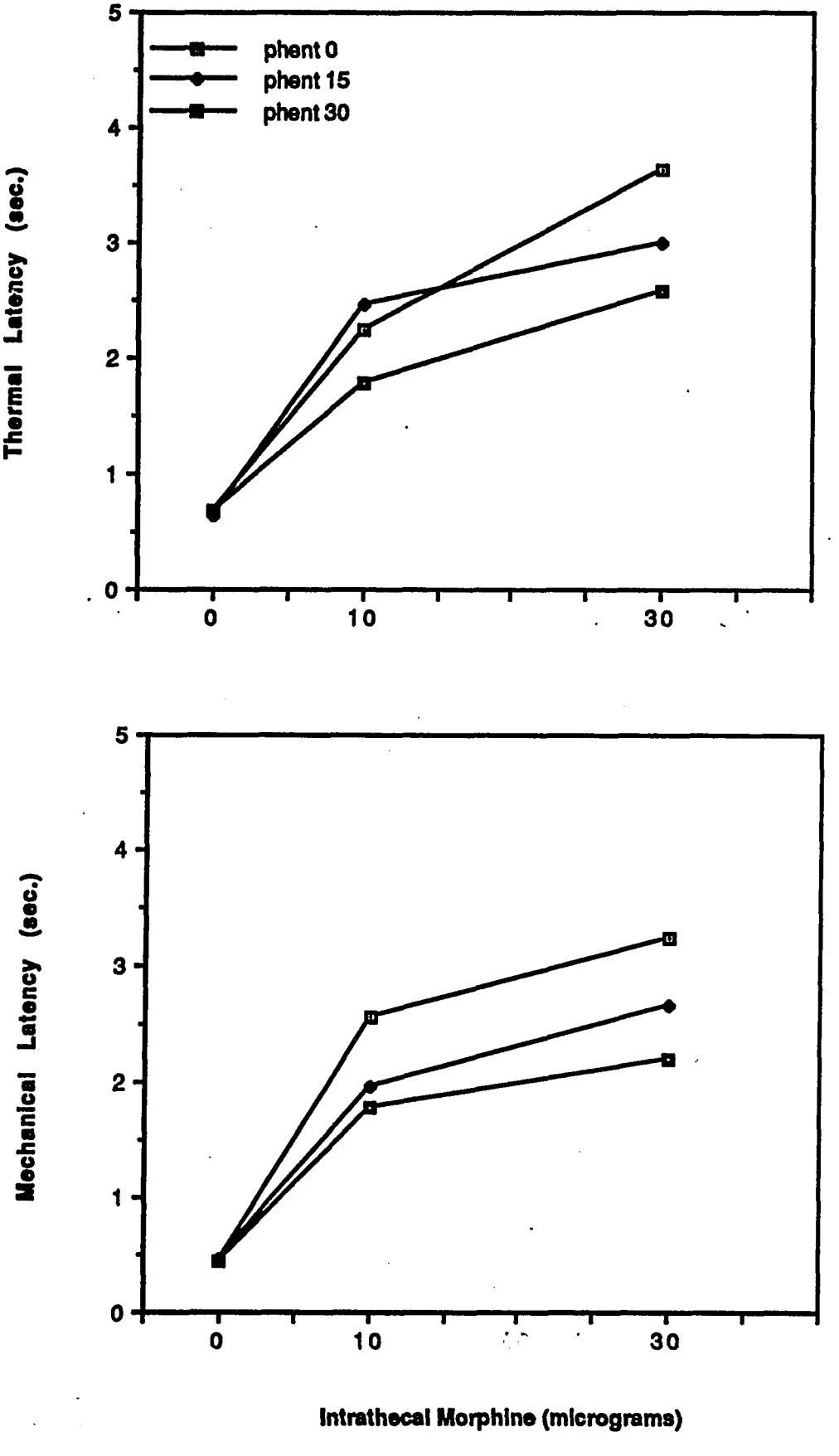

phent 0
phent 15
phent 30
Thermal Latency (sec.)
Mechanical Latency (sec.)
0
1
2
3
4
5
0
10
30
Intrathecal Morphine (micrograms)

Table 3

Mean and Standard Error Mean for Each Value Plotted in Figure 10

Dose IT Morphine (μg) for Thermal Test

Dose IT Phentolamine (μg)	0	10	30
0	.67 ± .02	2.23 ± .23	3.64 ± .45
15	.65 ± .03	2.45 ± .61	3.00 ± .63
30	.67 ± .05	1.78 ± .65	2.59 ± .69

Dose IT Morphine (μg) for Mechanical Test

Dose IT Phentolamine (μg)	0	10	30
0	.45 ± .03	2.57 ± .27	3.24 ± .39
15	.47 ± .03	1.95 ± .54	2.66 ± .78
30	.44 ± .04	1.78 ± .68	2.22 ± .39

Discussion

Experiment 2 indicates that morphine applied to the brain ventricles of preweanling rats resulted in elevated withdrawal latencies against thermally and mechanically noxious stimuli. No difference in the analgesic effects of morphine was observed between stimulus types. The more potent analgesia found against a mechanical rather than thermal stimulus when morphine was microinjected into the nucleus reticularis gigantocellularis of adult rats (Takagi et al., 1977; Kuraishi et al., 1980) was not found in infant rats with our injection procedure. Also, no difference in the effects of intracerebroventricular morphine was observed between 3 and 10 day olds. This latter observation raises questions regarding the developmental onset of opiate induced analgesia as indicated by previous studies in which a significant analgesic response to morphine first occurred at 6 (Spear et al., 1985) or even 14 (Barr et al., 1986) days of age. Zhang and Pasternak (1981) found that the dose of morphine required to produce thermal analgesia in 2 day old rats was 40 times greater than in 14 day olds. This weak analgesic response at 2 days of age was observed even though morphine gains easier access to the brain in younger rats (Kupferberg and Way, 1963). These developmental studies employed thermal algesic tests to determine the onset of morphine induced analgesia. Had mechanical tests also been used, ontogenetic differences in behavior as a function of stimulus type might have been assessed. Also, the age at which opiate mediated analgesia emerges is determined in part by the class of receptor agonist, drug dose and route of administration, length of post-injection period, stimulus intensity, site of stimulus application, and overall sensitivity of the behavioral assay used to measure pain. The systemic route of morphine administration used in the above mentioned studies might be expected to influence analgesic parameters differently than a morphine bolus injected directly to the brain. It is conceivable that opiate binding sites not accessed by systemic morphine due to loss of drug to nonspecific sites, could be

activated when the drug is concentrated into brain ventricles. An earlier behavioral onset might then be indicated in a developmental study using such a procedure. These data underscore the need to be cautious when comparing results from behavioral studies whose methodologies employ different pharmacologic criteria.

Intrathecal phentolamine antagonized the analgesic effects of intraventricular morphine against a mechanical but not thermal stimulus in both 3 and 10 day old rats. This is consistent with results from Experiment 1 and previous studies (Kuraishi et al., 1983, 1985) discussed in the "Mechanical vs Thermal Stimulus" section of this thesis. In Experiment 1, NE and clonidine applied to the spinal cord resulted in a dose dependent analgesia in the tail and hindpaw against a mechanical stimulus at 3 days of age. These agonists did not produce analgesia against a thermal stimulus until 7 days of age. In the present study, response latencies elevated by intraventricular morphine were lowered by intrathecal injections of a NE antagonist when tested with a mechanical but not thermal stimulus. This effect was also observed at 3 days of age. At 10 days, antagonism of morphine induced analgesia by phentolamine against a thermal stimulus was not observed. Compared to 3 day olds however, thermal response curves for each dose of phentolamine began to spread out in a dose dependent direction at 10 days suggesting that antagonism of the thermal morphine effect by phentolamine begins to emerge at about this age.

These data suggest that morphine sensitive brain sites activate noradrenergic cells that descend to the spinal cord and modulate afferent signals evoked by a mechanically noxious stimulus. Recently, Jensen and Yaksh (1986) investigated the extent to which opiate receptor-linked systems in the brain regulate reflexive pain responses by the co-activation of pharmacologically distinct spinopetal systems. They microinjected morphine into three brainstem nuclei of adult rats and observed thermal analgesia. Antagonists for NE (phentolamine), serotonin (methysergide) and dopamine (cis-

flupenthixol) were intrathecally administered and the degree to which analgesia was blocked was observed. They found that antagonists for both NE and serotonin blocked analgesia that resulted from morphine injected to PAG. The inhibition of spinal processing evoked by morphine applied to the nucleus paragigantocellularis was mediated by spinal NE while serotonin appeared to be important in processing the effects of morphine applied to the nucleus raphe magnus. Spinal application of cis-flupenthixol was without effect. This study permits speculation as to the relevant brain areas accessed by the intraventricular injection procedure used in the current thesis. The opiate receptor-linked systems activated by intraventricular morphine and whose effects were antagonized by intrathecal phentolamine might be located within the mesencephalic PAG or the medullary paragigantocellularis. Speculation is limited however by at least three observations: a) The Jensen and Yaksh (1986) study did not use a mechanically noxious stimulus. An important proposal of the current thesis is that analgesia against a mechanical stimulus is mediated primarily by a noradrenergic spinopetal system. Would differences in the degree of analgesia blockade by different spinal antagonists have occurred in the adult study as a function of stimulus type? b) Microinjection of morphine into specified brain areas would be needed to determine the specific opiate receptor systems involved in the current developmental study. By comparison, intraventricular injection is a much less circumscribed technique. It is likely that many if not all brain opiate systems are accessed by this procedure. c) The ontogeny of specified brain nuclei in 3 day old rats remains largely uninvestigated.

Morphine applied directly to the spinal cord of 10 day old rats resulted in elevated withdrawal response latencies when tested with both a thermal and mechanical stimulus. This effect is consistent with adult studies in which intrathecal morphine affected the latencies of a number of spinally and supraspinally organized responses associated with pain such as tail-flick in mice (Piercey, Lahti, Schroeder, Einspahr and Barsahn,

1982a; Piercey, Varner and Schroeder, 1982b) and cats (Yaksh, 1978a,c), pressure applied to paws in rats (Kuraishi et al., 1980), shock titration in rats (Yaksh and Rudy, 1977) and primates (Yaksh, 1983), shock induced vocalization in rats (Tang and Schoenfeld, 1978) and duration of time to report analgesia in humans (Bromage, Camporesi and Leslie, 1980; Nordberg, 1984). As intrathecal opiate effects have not been studied previously in preweanling rats, no precedent exists by which to directly compare the developmental aspects of the current findings.

Determining classes of interactions produced by cells containing different neurotransmitters is an ongoing process dependent on anatomic, biochemical, electrophysiological, pharmacological and behavioral corroboration. Inferences drawn from changes in behavior following drug administration can be applied to neuroanatomical concepts (i. e. type of synapse) only in a most abstract and tentative way. Any discussion of CNS structure based on results from this or related behavioral studies is speculative and offered only as perspective. Whereas a supraspinal opiate-NE link is supported by analgesia studies, the picture is less clear in the spinal cord. Results from the current study show that the analgesic effects of morphine applied to the spinal cord were not attenuated by intrathecally administered phentolamine. This finding is consistent with those of Russell and Yaksh (1981) that demonstrated that intrathecal phentolamine had no effect on response thresholds elevated by spinally applied morphine in adult rats. If opioid containing cells form synaptic connections with NE containing terminals in the dorsal horn, intrathecal phentolamine would be expected to reverse analgesia induced by morphine applied to the spinal cord. This result occurred neither in the adult study indicated above nor in the current thesis. As stated previously, anatomic studies using autoradiography and immunocytochemistry have not revealed enkephalinergic cells that synapse with NE terminals in the dorsal horn, though an enkephalin-serotonin link has been characterized (Glazer et al., 1981; Basbaum et

al., 1982). If NE containing cells form synaptic connections on opioid interneurons, naloxone would be expected to reverse analgesia induced by NE applied to the spinal cord. Studies using systemic naloxone reported no antagonism of analgesia produced by intrathecal NE (Reddy et al., 1980; Reddy and Yaksh, 1980). However, intrathecal naloxone has been shown to lower tail-flick latencies elevated by NE applied to the spinal cord (Loomis et al., 1987). The results of this thesis indicate that although both noradrenergic and opiate receptor activity in the spinal cord contribute to analgesia, these receptor populations appear to operate independently of each other. The ability of intrathecally applied phentolamine to antagonize the analgesic effects of morphine applied to the brain but not to the spinal cord suggests the presence of opioid-NE links at the supraspinal but not spinal level.

The application of receptor-specific drugs to select areas of the CNS in awake animals provides a useful means of understanding the pharmacology of systems that contribute to pain related behaviors. However, this procedure provides few clues as to what naturally occurring events activate these systems or how a reduction in pain sensation translates into an optimal behavioral strategy. A growing body of evidence indicates that certain treatments tentatively labeled "stressful", share the ability to induce analgesia that in some instances is naloxone reversible. Such treatments include centrifugal rotation, hypertonic solution, footshock (Hayes, Bennet, Newlon and Mayer,1978; Mayer and Watkins, 1981), tailshock (Woolf, Mitchell and Barrett, 1980), restraint (Amir and Amit, 1978), hypoglycemia (Bodnar, Kelly and Glusman, 1979; Bodnar, Kelly, Mansour and Glusman, 1979) and hypertension (Dworkin, Filewich, Miller, Craigmyle and Pickering, 1979). Not all of these stimuli are necessarily stressful and not all forms of stress induce analgesia. Also, whether or not the decreased sensitivity to pain following footshock is naloxone reversible may depend on such factors as stimulus duration (Lewis, Cannon and Liebeskind, 1980), area of application (Watkins and

Mayer, 1982), stimulus intensity (Cannon et al.,1984) and ability to control escape (Grau, Hyson, Maier, Madden and Barchas, 1981; Maier, Drugan and Grau, 1982). These concerns are well beyond the scope of this thesis. However, it must be noted that the subjects used in the current study were exposed to conditions that could be considered stressful, such as separation from the dam and siblings, handling, surgery under anesthesia, post surgical distress and possible food deprivation. Although yogurt was provided for all subjects, it is unknown whether each rat pup actually consumed the food. Therefore, the extent to which test results reflected drug action apart from general stress factors remains undetermined.

In conclusion, the receptor related, pharmacologic profile for opiate-NE interactions in regulating pain responses observed in the adult rat appears to develop early. Some investigators found a difference between thermal and mechanical analgesia when morphine was applied to the brain, while no such difference was observed at 10 days of age in the current study. Differences in morphine injection procedures between adult and infant studies could account for differences observed with respect to thermal vs mechanical analgesia. However, it is also possible that the neural circuitry that would account for a stronger opiate induced, analgesic response against a mechanical stimulus is not yet developed at 10 days of age. The evidence for involvement of spinal α-noradrenergic receptors in modulating pain signals evoked by a mechanical stimulus is consistent between adult and infant studies. This thesis has shown that intrathecal α_2-noradrenergic agonists produce analgesia in young rats that is more pronounced against a mechanical than thermal stimulus. Analgesia produced by intraventricular morphine is antagonized by intrathecal phentolamine against a mechanical stimulus as early as 3 days of age. The inability of phentolamine to antagonize the analgesic effects of spinally applied morphine in young rats is consistent with adult studies. Had an opiate-NE interaction occurred in young rats that was shown to be absent in adults, an ontogenetic

shift in the functional mechanisms involved in the regulation of pain might have been indicated. The search for such changes is not frivolous. Inhibitory pathways might function in the infant rat which are absent in the adult. Garcia-Arraras, Murakoshi, Yanagisawa and Otsuka (1986) have reported one type of descending inhibition of the monosynaptic reflex present in the neonatal rat that disappears as the animal matures. Certainly the difficulties involved in providing adequate pain control for human infants, who unlike adults cannot verbally report the presence, intensity or type of noxious stimuli, underscore the need to investigate the ontogeny of neural substrates that underlie pain sensation.

General Summary

The investigation of brain and medullary control of afferent pain signals at the spinal level has been a multidisciplinary effort. The immediacy with which pain as a clinical phenomena claims our attention as researchers is reflected in the medical community's overwhelming plurality of pain related cases. By comparison, the investigation of neural pathways that subserve other tactile senses is slight. This bias towards pain sensation as a field of study is sometimes reflected in the interpretations of the results that these studies generate. Sufficient evidence exists from this and other behavioral studies to warrant use of the term "analgesia" when considering response latencies in spinally treated animals. It would be "noci-centric", however, to conclude that the anatomically and cytochemically complex, spinal dorsal horn represents an area whose convergent inputs support a model for an endogenous analgesia system. It has been suggested by Dubner (1985) that descending monoaminergic cells serve the broader function of integrating pain related neural activity with other sensory input to permit maximally efficient behavioral responses. Segmentally located inhibitory cells containing GABA and enkephalin may serve to enhance features of the noxious stimulus through mechanisms based on signal to noise ratios. Aspects of behavior such as attention, stimulus relevance, and task-relatedness might be subserved by such neural mechanisms. Noradrenergic output from nucleus locus coeruleus has been implicated in the enhancement of behaviorally important signals in the cerebral cortex and cerebellum (Foote, Bloom and Aston-Jones, 1983). That descending NE-containing cells perform comparable functions at the spinal level should not be ruled out. Also, it has been demonstrated that inhibition of small, C and Aδ afferents effectively converts wide-dynamic-range spinal neurons into modality specific cells that respond selectively to Aβ input (Yaksh and Hammond, 1982). It seems likely that noradrenergic input to the dorsal horn serves more to regulate sensory processing by altering properties of

polymodal neurons to code for different sensations in response to a changing environment, than to specifically attenuate pain sensation (Yaksh, 1986).

In summary, the results of this thesis consist of the following conclusions: Withdrawal response curves using a range of thermal and mechanical stimulus intensities showed that reflexive pain responses can be reliably quantified in the developing rat. Soluble drug can be applied directly to the spinal cord and brain ventricles of awake, infant rats. The absence or attenuation of withdrawal responses following drug administration can be safely termed "analgesia" as indicated by the animal's ability to function motorically, by it's apparent ability to respond to other stimuli, and by it's apparent level of alertness. Drug, through the rupture of small blood vessels, does not reach the brain by the vascular system in sufficient quantity to affect analgesic responding. Possible intensity mismatches between algesic testing procedures or between animals of different ages do not account for differences in the shape of withdrawal response curves. Using a range of stimulus intensities, these curves showed that differences between thermal and mechanical responding in treated rats were due to drug-receptor interactions and not to possible intensity differences between the stimulus types. Manipulating the stimulus intensity did not cause the thermal and mechanical response curves to approximate each other. Intrathecal administration of the noradrenergic α_2 receptor agonists NE and clonidine produced a dose-dependent analgesia against a mechanical stimulus at 3 days of age. This effect was more pronounced in the tail and hindpaw than in the forepaw and was greater than the thermal effect at all ages tested. The difference between thermal and mechanical responding in the tail and hindpaw diminished as the animal matured. Clonidine produced analgesia against a thermal stimulus beginning at 7 days of age. This effect was the same for all three appendages. Clonidine's analgesic effects peaked at 10 days of age which correlates with peak noradrenergic receptor development as measured by the accumulation of NE-stimulated cyclic AMP in whole rat spinal cord at different

ages. The ability of spinally applied, noradrenergic receptor agonists to induce analgesia appears to develop in a caudal to rostral direction. Intrathecally administered phenylephrine was without effect against either stimulus at any age tested. Thus, the minor role of the spinal noradrenergic α_1 receptor in adult pain regulation is afforded no greater importance in the developing rat. Morphine applied to brain ventricles resulted in a dose-dependent analgesia on both thermal and mechanical algesic tests as early as 3 days of age. Intrathecal injection of the noradrenergic α-antagonist phentolamine reduced the magnitude of this effect against a mechanical but not thermal stimulus also as early as 3 days of age. Morphine applied to the spinal cord resulted in a dose-dependent analgesia on both thermal and mechanical tests at 10 days of age (the only age tested). Intrathecally applied phentolamine was without effect in altering response latencies elevated by spinal morphine. No significant limb effects were observed in these opiate-NE interactions. The analgesic effects of morphine applied to the brain appear to be mediated in part by the centrifugal activation of noradrenergic cells rather than by presynaptic, opioid activation of NE containing terminals in the spinal cord. Thus, the pharmacologic profile for opiate-NE interactions in modulating pain sensation observed in adult rat studies appears to develop early.

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