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THE ONTOGENY OF NOCICEPTION AND ANTI-NOCICEPTION IN THE FETAL AND INFANT RAT SPINAL CORD USING FOS PROTEIN AS A MARKER

by

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A dissertation submitted to the Graduate Faculty in Psychology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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Abstract

The Ontogeny of Nociception and Anti-nociception in Fetal and Infant Rat Spinal Cord Using Fos Protein as a Marker

by

Duckhyun Kim Yi

Advisor: Professor Gordon A. Barr

The first experiment examined the maturation of nociceptive primary afferents in awake 0, 1, 2, 3, and 14 day old rats. Pinch, immersion in hot water, or formalin injection was applied to the hindpaw. On the day of birth, all 3 stimuli elicited Fos immunoreactivity in dorsal horn cells. Fos expression was age and stimulus type dependent. The second experiment examined the appearance of Fos protein in response to formalin injection in fetal day (FD) 19, 20 and 21 animals. Very few cells showed Fos at FD 19, and the number of Fos nuclei first appeared in significant numbers at fetal age 20. There was a large increase in the number of Fos labeled nuclei between FD 20 and 21.

Experiment 3 examined the inhibition of formalin- induced Fos immunoreactivity in the spinal cord following different anesthetic treatments: a mixture of xylazine and ketamine, methoxyflurane, acepromazine and hypothermia. All treatments induced behavioral anesthesia. Despite the anesthesia, the ketamine-xylazine mixture was completely ineffective in suppressing Fos immunoreactivity. In contrast, methoxyflurane and hypothermia blocked the appearance of Fos protein

following formalin injection. Mechanisms by which these agents produce anesthesia are discussed.

The effects of ICV morphine administration on behavior and Fos immunoreactivity in the spinal cord following formalin injection or application of noxious heat in the paw pad of 3 and 14 day old rats were studied. Pups injected with formalin showed profound analgesia in both the forepaw and hindpaw following ICV morphine administration whereas in the thermal test, the hindpaw was never analgesic. However, ICV morphine administration reduced the number of Fos stained nuclei in both the formalin and thermal test. The behavioral data suggest that development of analgesia is a function of both stimulus types and the body part stimulated. The Fos data suggest that mechanisms by which morphine is producing its analgesic effects, such as descending inhibitory control system, are immature during the early postnatal period.

Taken together, these results suggest that 1) nociceptors are functional before birth and undergo significant postnatal maturation. 2) the discrepancy between behavior and Fos suppression exists both during pain processing and dampening. 3) Fos expression is not a good predictor of behavioral analgesia, and 4) discrepancy between Fos immunoreactivity and behavior is exaggerated depending on the type of noxious stimulus.

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I dedicate this work which took 2 years short of a decade to my father, Mr. Myung Soo Kim for it was his blind trust in me that motivated me to pursue this degree and more importantly to complete it.

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CHAPTER ONE:

General Introduction

Until about a decade ago, invasive medical procedures such as circumcision or repeated venepuncture in newborn patients were routinely performed without any anesthetics or analgesics. Reactions that infants displayed during and following these procedures including crying, withdrawing from the source of a noxious input and face grimacing were considered to be stress or non-specific distress responses, not behaviors specific to pain (Craig, Whitfield, Grunau, Linton, & Hadjistavropoulos, 1993). Many pediatricians and anesthesiologists believed that infants did not feel or remember pain because the infant's immature central nervous system lacked myelination and dendritic arborizations. In addition, the fear of side effects of opiate drugs including respiratory and cardiovascular depression, prevented or, at best, limited the proper medication of infant patients. The evidence now suggests that infants do feel pain and are even profoundly altered by the experience (Andrews & Fitzgerald, 1994). A detailed understanding of how nociceptive input is processed and modulated in the immature animal is the necessary first step in controlling and alleviating pain more effectively in neonates.

In the past decade, the expression of immediate-early genes (IEG) such as c-*fos* has been used to mark activated neurons. C-*fos* gene expression in particular has been used widely in pain studies because neurons that are known to be nociceptive express Fos protein following a noxious input. In this thesis, immunocytochemical visualization of Fos protein is used as a marker of neuronal activity in response to primary afferent activation. The overall goal of this thesis is to examine ontogeny of nociception and antinociception using anatomical and behavioral approaches. Specifically, this thesis examined first, the developmental profile of functional

maturity of primary afferents. Second, I examined whether primary afferents and nociresponsive neurons are modulated by different pharmacological manipulation. Third, the functional maturity of the descending inhibitory control system at different levels of the cord was examined. In addition to examining behavior, Fos expression in the spinal cord was quantitated and analyzed. This dissertation concentrates on examining events in the spinal cord, the location of first central synapse of the nociceptive circuitry. Understanding what happens at the spinal level is a necessary first step, and an essential one in attempting to understand operation of pain circuitry at higher level of the neural axis (Fitzgerald, 1993).

The following sections review the literature on the functional and anatomical development of pain pathways. Most data in this area are obtained using the laboratory rat and the literature that is reviewed, unless otherwise noted, is mainly of that species.

The Anatomy of developing pain pathways.

Nociceptors.

Noxious information is transmitted to the central nervous system from the periphery by two types of primary sensory afferents: unmyelinated C-fibers and thinly myelinated Ad fibers. There are two types of A fibers: the Ad fibers, high threshold mechanoreceptors and the A-a/b fibers, low threshold mechanoreceptors.

Dorsal root ganglion cells are born between gestation day (GD) 11 and GD15 in the rat (Lawson et al., 1974). Fibers originating from lumbar region of the dorsal root ganglion innervate the paw by GD 14.5 - 15, and the epidermis of the most

distal toes is innervated by GD 16 - 16.5 (Mirnics and Koerber, 1994). The central projection of these fibers follows similar time course as the peripheral projection. Dorsal roots grow toward the thoracic and lumbar cord between GD13.5-14.5 (Altman and Bayer, 1984; Smith, 1983; Fitzgerald, Reynolds and Benowitz, 1991). On GD 15 - 15.5, primary afferent axons begin to enter the superficial layer (laminae I and II) of the thoracic (Smith, 1983) or lumbar spinal cord (Fitzgerald et al., 1991; Mirnics and Koerber, 1994), and by GD 16.5-17.5, afferents from the muscle and low-threshold cutaneous A fiber afferents terminate in the intermediate gray matter near the central canal (lamina X) and deep dorsal horn, respectively. In addition, sensory afferents innervating the proximal and distal hindlimb entered the superficial layer of the dorsal horn simultaneously at GD 15 (Mirnics and Koerber, 1994). The sensory afferents enter the spinal gray matter at GD 15 with the nociceptors being the last population of afferent fibers to enter the dorsal horn, very late in the fetal life. Ad and polymodal C-fibers begin to penetrate the dorsal horn on GD19 and reach superficial dorsal horn on GD19.5 -20 (Fitzgerald,. 1987a). Some Ad fibers synapse in deeper layers of dorsal horn and therefore it is likely that they penetrate dorsal horn earlier than the C-fibers (Fitzgerald, 1987a). At birth, the density of Ad and C-fibers is similar to that observed in the neonate and young rat, but an electron microscopic study revealed that a normal C-fiber terminal is seen only after postnatal day (PD) 5 (Pignatelli et al., 1985).

In the adult, primary afferent fibers termination on the second order neurons represents an exact somatotopic map of the body. Moreover, primary afferent fibers that are innervated by different peripheral receptors project to specific laminar locations in the spinal cord. In the maturing animal, fibers carrying different

sensory modalities grow into their exact somatotopic location in the cord (Fitzgerald and Swett, 1983; Smith 1983) with no exuberant projections. Muscle afferents project to the intermediate gray matter and ventral horn whereas cutaneous afferents carrying both noxious and non-noxious information terminated mainly in the dorsal horn. The low threshold cutaneous A fiber terminals extend into the superficial layers although their final target is restricted to laminae III-V (Fitzgerald et al., 1994).

In summary, peripheral projection of afferent fibers is completed before the central innervation at GD 16.5. The central projection of these fibers occur in a somatotopic and modality specific manner, with the nociceptors being the last population of afferents, entering the spinal cord, at GD 19.

Dorsal horn neurons.

In the adult, Rexed's laminae I and II receive inputs from polymodal C-fibers and laminae I, II and V of the spinal cord receive input from Ad nociceptors. However, it is important to note that no single spinal cord region is exclusively innervated by nociceptors (Light, 1990). Generally, in addition to receiving inputs from myelinated (Ad fibers) and unmyelinated nociceptors (C-fibers), lamina I also receives inputs from innocuous thermoreceptors. A small percentage of inputs into lamina II is from nociceptors, but the majority of inputs are from innocuous mechanoreceptors. Lamina V receives inputs from myelinated nociceptors and myelinated mechanoreceptors.

Neurons in the spinal cord are generated between GD 11 and 16 (Nornes & Das, 1974). Neurogenesis in the spinal cord occurs in the rostral to caudal direction: neurons in the cervical portion of the cord are born first, followed by neurons in the thoracic and lumbar regions (Nornes & Das, 1974). Within each segment of the cord, neurogenesis starts in the ventral horn and moves dorsally in a systematic manner. In the spinal cord of the rat, there are 3 categories of neurons: large-sized motor neurons, medium-sized and small-sized cells. The large-sized motor neurons originate between GD 11 and 13, with the more rostral region of the spinal cord starting neurogenesis on GD 11. The next population of neurons to initiate neurogenesis is medium-sized neurons located in the ventral horn, in the intermediate gray region, and in the dorsal horn including the basal portion of the substantia gelatinosa. These neurons originate between GD12 and 15, with cells found in the cervical portion of the cord undergoing neurogenesis earlier than cells in the lumbar region. The last population of spinal cells to be born is the small-sized neurons found mainly in the substantia gelatinosa (lamina II). The small-sized neurons are also found in the upper dorsal horn area, and these cells originate between GD 12 and 15 following the same temporal sequence as the medium-sized cells. The small neurons in the substantia gelatinosa appear during the final stages of spinal cord neurogenesis, between GD 14 and 16. There are two populations of neurons in the substantia gelatinosa, projection neurons with long axons and non-projection intrinsic interneurons (Bicknell & Beal, 1984; Fitzgerald et al., 1991). The time course of maturation of these two populations of neurons differs. Axonal and dendritic development of the projection neurons occurs between GD15-21, and by birth, maturation is nearly complete. By postnatal day 10, these neurons

resemble those in the adult (Bicknell & Beal , 1984; Fitzgerald et al., 1991). On the other hand, the interneurons do not initiate their axonal and dendritic maturation until just before birth, GD 21, and continue to mature well into the postnatal life (Bicknell & Beal, 1984). By postnatal day (PD) 20, mature interneurons are found in substantia gelatinosa.

Neurochemistry

In the adult, several peptides are involved in nociception: substance P (SP), calcitonin gene-related peptide (CGRP), somatostatin, vasoactive intestinal polypeptide (VIP), and galanin (Gibson, Polak, Bloom & Wall, 1981). Fluoride-resistant acid phosphatase (FRAP), an enzyme, and excitatory amino acids such as glutamate and aspartate also participate in nociception (for review, Dickenson, 1995). These neurochemicals are found in the dorsal root ganglion cells, terminals of the primary afferent fibers or in the dorsal horn neurons of both projection and non-projection neurons (Dodd, Jahr & Jessell, 1984). SP and somatostatin are found in the terminals of unmyelinated small-diameter fibers (Hunt & Emson, 1981), and FRAP is found in a discrete band in the substantia gelatinosa. The function of some of these peptides and excitatory amino acids are well known, but others are less well described. For example, somatostatin, the only potential primary afferent inhibitory transmitter, is released in the dorsal of horn following noxious input and causes hyperpolarization of dorsal horn neurons (Dickenson, 1995). In contrary, the exact mechanisms by which CGRP or galanin participate in the pain circuitry, or the role of FRAP during pain processing are still largely unknown.

A small amount of FRAP is found starting GD 9, but a strong FRAP activity is observed in dorsal root ganglion cells and motor neurons between GD 13 and 15 (Schoenen, 1978). FRAP activity is not detected in the dorsal horn of the fetal animal (Schoenen, 1978), but is located in the spinal cord within 12 hours of birth (Fitzgerald and Gibson, 1984; Mattio, Rosenqvist and Kirby, 1981), and reaches the adult concentration of FRAP during the first week of postnatal life (Fitzgerald & Gibson, 1984). Somatostatin is observed in the ventral and dorsal horn of the spinal cord at GD 15. Somatostatin-positive cells in the superficial layers of the dorsal horn increase in number as the fetus matures. After birth, however, the number of somatostatin-positive cells in this area decreases and an adult-like distribution is apparent by PD 14 (Marti et al., 1987, Senba et al., 1982). SP is observed in the spinal cord between GD 15 and 18 (Marti et al., 1987; Senba et al., 1982), and the number of SP-immunoractive cells decreases in number towards parturition. SP reaches the adult concentration by PD 10 (Marti et al.,1987; Senba et al., 1982). Fibers containing CGRP, and galanin are observed in the dorsal horn between GD 16 and 18 with a marked increase in the number of immunoreactive fibers during perinatal period (Senba et al.,1982; Marti et al., 1987; Fitzgerald and Gibson, 1984; Pickel, Sumal, and Miller, 1982).

The Physiology of developing pain pathway

Nociceptors.

In the adult, unmyelinated C-fibers respond to skin deformation, and noxious heat (heat greater than 45^{o} C), and chemical stimulation (such as acids, bradykinin, and methyl chloride). Because these nociceptors respond to a variety of stimulus

types, they are called C-polymodal nociceptors. In contrast, myelinated Ad fibers respond only to noxious skin deformation and are accordingly called high threshold mechanoreceptors. Fetal dorsal root ganglion cells respond to noxious thermal, mechanical and mustard oil applied to the hindpaw starting at GD17 although frequency of firing and total number of impulses per stimulus is low compared to the newborn or adult, and, unlike in the adult, there was no correlation between conduction velocity and receptor types (Fitzgerald, 1987c). These results suggest that the peripheral terminals have defined receptive fields and respond to touch, pressure, noxious heat and chemical stimulation prenatally. By birth, the polymodal nociceptors respond to intense mechanical stimulation, noxious heating of the skin (50 °C) and noxious chemical stimulation and they respond with the same firing frequencies and pattern as those found in the adult (Fitzgerald, 1987a). In contrast, however, high threshold mechanoreceptors respond at lower frequencies at birth than in the adult.

Dorsal horn cells.

Fetal dorsal horn cells respond to electrical stimulation starting GD 17, but they do not respond to pressure or pinching of the hindpaw until GD 19 (Fitzgerald, 1991). At birth, cells in laminae I, II, and III respond to light brushing and pinching (Fitzgerald, 1985), but cells in deeper layers of dorsal horn do not respond to C-fiber activation until the second week of life in the rat (Fitzgerald, 1985; 1988). In contrast, cells in these regions do respond to A fibers. These results suggest that development of responses evoked by C-fibers in the deeper dorsal horn is delayed compared to those of responses evoked by A fibers.

Development of reflex responses to nociceptive stimuli

Cutaneous flexor reflexes reflect the properties of cells in the dorsal horn and sensory interneurones that are involved in mediating and modulating somatosensory and nociceptive signals. They provide a useful measure of nociceptive function of the central nervous system in adult humans and rats (Woolf, 1983). In the adult rat, the flexor reflex activates exclusively nociceptive projection pathways to the brain (Cook and Woolf, 1985). However, in the immature animal, the flexor reflex can be activated by low-threshold inputs, and therefore, it may not be an exclusive measure of nociceptive function as it is in the adult. The occurrence of flexor reflex following non-noxious input is suggestive of allodynia, where innocuous stimuli are perceived as painful (Fitzgerald, 1995). Forelimb cutaneous reflexes can be evoked in the rat on GD 16 (Narayanan et al., 1971). Hindlimb cutaneous reflexes develop later on GD 17-18 (Narayanan et al., 1971), consistent with rostrocaudal development of the CNS. At birth, flexor withdrawal reflexes to pinching and heating the skin of the hindpaw are exaggerated in amplitude and duration compared to the adult response (Fitzgerald and Gibson, 1984) and because the reflex is elicited by only light touch, flexor reflex thresholds are very low (Fitzgerald et al., 1988). Flexor reflexes to the irritant chemical, mustard oil, are not present until day 10-11 of life (Fitzgerald and Gibson, 1984). Injection of formalin, another chemical irritant, into the paw pad produces responses as early as on the day of birth (Guy & Abbott, 1982), and paw licking and lifting by 3 days of age in rats (McLaughlin et al., 1990). Formalin injection is extensively used in pain research as a stimulus that produces tonic nociception. In the adult, formalin activates exclusively the C-fibers

(Dickenson and Sullivan, 1987), but in the immature animal, it may act as a multimodal irritant (Fitzgerald, 1995).

The literature on the development of pain circuitry is small, and particularly, little is known about the functional maturity of the nociceptors and the spinal neurons on which these afferent fibers terminate. The available data on anatomy, physiology and neurochemistry of the developing pain pathway indicate that, despite early anatomical presence of the system, both the neurochemistry and functional maturity of nociceptors and nociresponsive cells in the spinal cord undergo considerable maturation in the first 3 weeks of rat's postnatal life.

Development of Opiate Induced Analgesia.

Analgesia occurs in immature rats and the adult pattern of analgesia is observed by the first 3 weeks of rat's postnatal life (Auguy-Valette et al., 1978; Pasternak et al., 1980; Zhang and Pasternak, 1981; Barr et al., 1986; Giordano and Barr, 1987; Tive and Barr, 1992). However, the specification of its exact developmental profile in the rat pup is dependent on such methodological considerations as the body part stimulated, the type and intensity of stimulus used, and the opiate that is given to induce analgesia (for recent review, Barr, 1992). When less intense stimulation is used or rostral body parts are stimulated, opiate-induced analgesia is observed at an earlier age than when more intense stimulus or when more caudal parts are stimulated (Giordano and Barr, 1987; Barr et al., 1992; Barr and Wang, 1993). For example, the forepaw is consistently more sensitive to opiates than are the hindpaws or tail (Barr et al., 1987; Giordano and Barr, 1987; Barr et al., 1992). This rostral to caudal development of analgesia is also dependent on the stimulus

type. For example, morphine-induced analgesia against the mechanical stimulus is observed in the hindpaw of 14 day old rat, but not against noxious thermal stimulation at this age. The apparent difference between mechanical and thermal stimuli has been attributed to different mechanisms by which these two stimuli produce nociception. In both adult and immature animal, noradrenergic and serotonergic system have been implicated in analgesia against mechanical and thermal stimuli, respectively. These results thus suggest that morphine interacts with different neurotransmitter systems in modulating pain processing, and further this interaction depends on the type of noxious stimulation. Although formalin injection is extensively used in pain studies as a model for inflicting tonic pain, particularly in studies using Fos expression, there are no available data that describe the development of opiate induced analgesia against formalin at different levels of the spinal cord.

Locus of opioid-induced analgesia

Opiates modulate transmission of nociceptive inputs at several sites in the central nervous system. These drugs can inhibit spinal cord neurons directly, inhibit transmission through ascending pain pathway in the brain, or by activating descending medullary-dorsal horn inhibitory projections (Basbaum and Fields, 1984; Lipp, 1991). At the spinal level, opiates inhibit the nociceptors by either the postsynaptic inhibition of the nociceptive projection neurons and or the presynaptic inhibition of primary afferent inputs. Consistent with these mechanisms, opioid receptors are found both in the presynaptic terminals of the primary afferents and in the spinal cord cells. There is a predominant number of opioid receptors sites on the

terminals of the primary afferent fibers; over 70% of the total mu sites are on the primary afferent terminals (Bess, Lomard, Zakac, Roques, and Besson, 1990). In the spinal cord, the highest levels of opioid receptors are found in the termination sites of the C-fibers in lamina I and the substantia gelatinosa, with a smaller number of receptors in the ventral horn. The mu receptors are the most common opioid receptors found in the spinal cord (for recent review see Dickenson, 1995). Opioids acting on the presynaptic sites prevent or reduce the amount of transmitters released by the primary afferents. The reduction of primary afferent transmitters is produced by hyperpolarization of the terminals that opens the potassium channels. Postsynaptic action of opioids is produced by hyperpolarizing the nociresponsive neurons in the spinal cord that in turn depresses the transmission of the painful signal.

At the supraspinal levels, medullary and midbrain nuclei have been implicated as the anatomical substrate for opioid analgesia. Microinjection of opiates (Murfin et al., 1976), or electrical stimulation (Reynolds, 1969; Basbaum et al., 1977) of the periaquaductal gray (PAG) area generates analgesia by ultimately activating the dorsolateral funiculus (DLF), which inhibits the firing of dorsal horn neurons (Liebeskind et al., 1973). In the adult, dorsolateral funiculus contains descending axons from the brainstem that inhibit dorsal horn cell activities. This descending pathway is a part of an endogenous analgesic system that dampens the effects produced by nociceptors at the spinal level. The rostral ventral medulla is the major source of axons projecting in the DLF to the spinal cord. Medullary cells that descend in the DLF originate in the nucleus raphe magnus (NRM) and the reticular formation. These descending inhibitory paths are thought to be serotonergic and

adrenergic (Kuraishi et al., 1983), and produce their antinociceptive effects by synapsing with opioid peptide containing neurons in the dorsal horn (Yaksh and Noueihed, 1985).

The descending inhibitory pathways in the pain system in developing animals are functionally immature at birth (Gilbert & Stelzner, 1979; Fitzgerald & Koltzenburg, 1986), and develop during the first 3 weeks of life in the rat. The inhibitory pathways in the DLF become functional at PD 10 and the adult pattern of response is observed by PD 21 (Fitzgerald & Koltzenburg, 1986). Despite their slow physiological maturation, axons descending from the brainstem are present on the day of birth (Lelong, Sheih, & Wong, 1984). The delay in functional maturity of this pathway has been attributed in part to late developing transmitters such as serotonin and norepinephrine in the terminals of these pathways (Fitzgerald & Koltzenburg, 1986). There are no monaminergic cells bodies in the spinal cord. Therefore, serotonergic and noradrenergic fibers innervating the spinal cord cells descend from the brain. Norepinephrine fibers are distributed uniformly in the spinal cord by PD 10, but serotonergic fibers innervate the more rostral level of the cord earlier than with more caudal levels. The adult pattern and density of serotonergic immunoreactivity is found by PD 14 and 21 in the cervical and lumbar spinal cord, respectively (Bregman, 1987).

The literature on the development of opioid-induced analgesia clearly demonstrate that drugs can induce analgesia in the immature animal during the neonatal period. However, the systems that mediate analgesia are highly immature

during the first 3 weeks of postnatal life. In particular, the descending inhibitory system is functionally immature despite its early anatomical presence.

The use of immediate-early genes in pain research

Immediate-early genes (IEG's) or primary response genes are a class of genes that are rapidly but transiently induced following extracellular stimuli such as seizures, hypothermia, light, inflammation and different forms of noxious stimuli. They are so-called because transcription of these genes occurs without the help of protein synthesis and therefore requires only the modification of pre-existing transcription factors. C-*fos* is a member of IEG's that is also a proto-oncogene. Proto-oncogenes are normal cellular genes believed to be substrates for many of the somatic mutations responsible for neoplasm. Activated or mutant proto-oncogenes are called oncogenes and are responsible for the production of tumor cells.

Induction of IEG's is likely play an important role in regulating the gene expression of target genes in response to extracellular signals. Extracelluar stimuli that induce the transcription of c-*fos* gene also induce c-*jun*, another member of IEGs. The mRNAs of the c-*fos* and c-*jun* genes accumulate within minutes following extracellular stimulation in the cytoplasm where these gene are translated. Fos and Jun, the protein products of c-*fos* and c-*jun*, respectively, are translocated to the nucleus (Morgan and Curran, 1989), where they form a dimeric complex that binds at the transcription factor activator protein-1 (AP-1) binding site of the target gene and alters the expression of that gene (Morgan and Curran, 1989). The protein products of c-*jun* or c-*fos* can be readily localized with immunohistochemical assays.

In general, the induction of c-*fos* gene is useful as a marker in response to extracellular events for the following reasons. The Fos protein and c-*fos* mRNA have low basal levels especially in the spinal cord, and the protein and mRNA have a short-half life. Therefore, any change in the levels of either the mRNA or the protein can be detected. Further, the c-*fos* mRNA can detected within minutes following stimulation and the maximum amount of Fos protein is observed at 2 - 4 hours after the extracellular stimulus. The transient and rapid appearance of these proteins make them useful as messengers for extracellular information. Measurements of the expression of c-*fos* gene can provide data that are not readily available through the use of other techniques and compliment data obtained by other methods. Electrophysiological techniques are used to examine function, but these procedures use anesthetized animals, and the number of cells that can be examined is extremely limited. Other metabolic markers such as 2-deoxyglucose (2-DG) autoradiography are technically difficult and cellular resolution can only be obtained with difficulty by analyzing the grains. Cytochrome oxidase has a cellular resolution, but the basal level of cytochrome oxidase activity is sufficiently high that it is difficult to detect change. Fos appearance can be visualized in individual cells, and the number of Fos labeled cell can be quantified and a population of activated neurons can be monitored. In addition, both behavior and activated neurons can be studied in the same animal.

In PC 12 cells, Fos and Jun are induced by neurotrophic factors, neurotransmitters and an influx of Ca^{2+}. In intact animals, c-*fos* expression is induced by pharmacological (Morgan et al., 1987), electrical (Dragunow & Roberts, 1987) and a variety of physiological stimuli. These stimuli include, among

others, dehydration (Sagar et al.,1988), light (Rea, 1989), hypothermia (Joyce and Barr, 1992), seizures (Morgan et al., 1987; Jones and Evinger, 1991), and pain (Bullitt, 1989; Menetrey et al., 1989; Bullitt, 1990; Presley et al., 1990; Williams et al., 1990a; Williams et al., 1990b; Bullitt et al., 1992). Following these stimuli, Fos expression is consistently found in the anatomically appropriate sites. The c-*fos* gene has been especially useful in examining pain circuits because noxious stimulation activates the c-*fos* gene in the known primary afferent termination sites; laminae I and II when C-fibers are activated, and laminae I, II and V for Ad-fibers stimulation (Hunt et al., 1987; Presely et al., 1990; Gogas et al., 1991). For example, Hunt and his colleagues (1987) showed that noxious heat or mustard oil can induce the expression of IEGs, c-*fos* and c-*myc*, in the spinal cord following noxious heat or mustard oil administration in the hindlimb. Most labeled nuclei were found in marginal (lamina I) and substantia gelatinosa (lamina II) where majority of unmyelinated nociceptors terminate and a few Fos positive cells were also found in lamina V, VI and VII and around the central canal (lamina X). This was the first study that showed that sensory information can influence gene expression in neurons of adult central nervous system. Numerous studies have since consistently shown that noxious stimuli produced labeled Fos nuclei in the anatomically appropriate areas of the cord. In general, cells in the ventral horn have not been associated with pain processing, but neurons in this region express c-*fos* gene following noxious stimuli (Presley et al., 1990). Intensities of stimulation has shown to be an important factor in the laminar distribution of Fos appearance (Bullitt, 1992). In somatosensory especially pain pathways, the functional role of c-

fos gene has been a subject of investigation, and those results are discussed next section.

Because c-*fos* gene expression is induced by a variety of stimuli in many different systems, target genes for the Fos and Jun complex may dependent on the system being studied. In pain circuitry, there is increasing evidence that Fos and Jun complex may be targeting the endogenous opioid genes. The endogenous opioid genes became candidates as the target genes for Fos protein because of increases in the levels of prodynorphin and proenkephalin mRNA's following peripheral inflammation and chronic arthritic pain (Hollt, Haarmann, Milan, and Hertz, 1987; Noguchi, Dubner, and Ruda, 1992). Subsequently, it has been shown that the Fos protein may regulate the transcription of preproenkephalin and preprodynorphin genes (Noguchi, et al., 1992; Noguchi, Kowalski, Traub, Soldkin, Iadarola and Ruda, 1991), the two genes of the opioid family found in the rat spinal cord (Hollt, 1983). In these studies, a dramatic increase in dynorphin or enkephalin peptides, and preprodynorphin or preproenkephalin mRNA's, following inflammation of the hindpaw by complete Freund's adjuvant or carrageenan (Draisci and Iadarola, 1989; Iadarola, Brady, Draisci and Dubner, 1988; Noguchi et al., 1991; Ruda, Iadarola, Cohen and Young, 1988) or formalin (Noguchi, Morita, Kiyam, Sato, Ono and Tohyama, 1989), was observed in neurons expressing Fos protein. These results are consistent with c-*fos* gene induction of opioid peptides synthesis in the same population of the cells. More recently, Hunter and colleagues (1995) provided compelling evidence that the c-*fos* gene may target one or more of the opioid genes. Administration of short antisense probes *in vivo* blocks the translation of the mRNA. Following intrathecal injection

of c-*fos* antisense oligodeoxynucleotide there were no increases in Fos immunoreactivity or preprodynorphin mRNA in the lumbar spinal cord neurons following formalin injection into the hindpaw. Taken together, these results suggest that there is increasing evidence that Fos protein may turn on opioid genes and the resulting opioid peptides may modulate nociception. Although this thesis does not address the issue of functional significance of c-*fos* expression, these data implicate a wider role of Fos protein in pain circuitry.

Goals of this thesis

Experiments in thesis were designed to examine pre-and-postnatal development of pain processing and the mechanisms by which morphine induces analgesia in the postnatal animal. The experiments in this thesis are unique because they examined age related changes in nociceptive pathway at the spinal cord level using the upregulation of an immediate-early-gene, c-*fos*. Moreover, although no attempt is made to correlate Fos expression with behavior directly, this is the first developmental study to provide both behavioral and functional maaturity of nociceptive pathway in the same animal.

The following hypotheses were tested in this thesis:

1. Formalin can activate primary afferents on or about GD 19, and the nociceptors mature as a function of age.
2. Four commonly used anesthetic agents may reduce formalin induced primary afferent activation in neonatal rats.
3. Centrally administered morphine induces analgesia by acting through the descending inhibitory pathway.

The first hypothesis was tested by injecting formalin directly into the paw pad of fetal animals at GD 19, 20, and 21. Both the forepaw and hindpaw were injected with formalin. By examining behavioral responses to formalin in the forepaw and hindpaw and Fos immunoreactivity in these animals, the functional maturity of the nociceptive pathway at different levels of the spinal cord was examined. Postnatal maturational status of the primary afferents was examined by administering noxious mechanical, thermal, and chemical in the paw pad at various postnatal ages. The second hypothesis was tested by administering different commonly used anesthetic regiments before the formalin injection. Reduction of Fos immunoreactivity in the spinal cord cells would suggest that mechanisms by which these regiments induced anesthesia may in part reside at the spinal level. If, however, no reduction in the number of Fos stained nuclei is observed, then, anesthetic agents may act on sites other than the spinal level. When morphine effects are limited to the central sites, it influences activities at the spinal level mainly through activating the descending inhibitory path. Therefore, the third hypothesis was tested by administering morphine directly into the brain, and reduction of Fos stained nuclei at different levels of the spinal cord was examined.

General Methods

Experimental Animals Subjects were awake Long-Evans hooded 0, 1, 2, 3, and 14 day old rat pups born and bred in our animal facilities. Dams were checked for new births twice daily at 9 AM and 5 PM. The day of birth was designated as age 0. All pups were housed with both parent animals and their siblings in plastic cages

measuring 40 X 20 X 24 cm with bedding and food and water available ad libitum. The animals were maintained under a 12-hour and 12-hour/light-dark cycle (lights on at 7:30AM) and the colony room temperature was 24±1°C. Pups were removed from the cage and kept warm (34 °C) in an incubator until stimulated. While the animals were being stimulated, they were placed on a heating pad to maintain normal body temperature. Animals were returned to the incubator after the end of stimulation period and remained in the incubator with their littermates until they were sacrificed. Because anesthesia has been reported to suppress Fos expression (Dragunow and Faull, 1989; Presley, 1990), this study was conducted in awake rats.

All experiments were approved by both Hunter College and New York State Psychiatric Institute IACUC's and followed ethical guidelines of the Society for Neuroscience and the International Society for Developmental Psychobiology.

Histochemistry Animals were injected intraperitoneally with an overdose of sodium pentobarbital (65 mg/kg). When deeply anesthetized, the animals were perfused transcardially with 0.1 M phosphate buffered saline (PBS), pH 7.2, followed by 4% paraformaldehyde. The spinal cord was removed and placed in 30% sucrose for at least 24 hours prior to sectioning.

Thirty micron transverse frozen sections of the entire lumbar spinal cord were cut and every third section was collected in 0.1 M PBS, pH 7.2. The sections were processed for immunocytochemistry by the avidin-biotin-peroxidase (ABC) method of Hsu et al. (1981). The free floating sections were washed with 0. 1 M PBS, pH 7.2, and were treated with 3% hydrogen peroxide for 3 minutes to quench

endogenous peroxidase activity. The sections were incubated in blocking solution of 10% normal goat serum for 20 minutes and then incubated overnight on a shaker at 4 °C in the primary antibody, rabbit-anti-fos (Oncogene Sciences, Manhasset, NY), diluted 1 to 1, 000 (v/v) in PBS with 2% BSA. On the following day, the sections were incubated with diluted biotinylated secondary antibody for 1 hour and in avidin-biotin-peroxidase complex (Vector Labs, Burlingame, CA) for another hour. Diaminobenzidine (DAB) was the chromogen.

Controlling inter-assay variability There are two major sources of inter-assay variability; variability due to experimental error such as differences in assay conditions, in section thickness, or the plane at which the sections were cut, and the actual difference in the concentration of antigens in the neurons. On the other hand, a large proportion of within-assay variability can be attributed to different concentration of protein present in different animals. In this thesis, many different sets of immunocytochemistry were performed for each experiment. Therefore, it was critical to control or reduce inter-assay variability that was due to experimental error. One way of controlling this type of error is by processing tissues from all the groups in the experiment together so that the "noise" can be kept constant across all the tissue. This was the strategy used in this thesis. For example, for chapter 5 formalin experiment, 12 different groups of animals (2 ages X 2 limbs X 3 morphine doses) were sectioned and assayed simulatneoulsy .

Data Analysis Lumbar sections (L1-L6) were examined under the light microscope, and labeled nuclei were counted using a drawing tube attachment. The spinal cord sections were subdivided into 5 different regions (Fig. 1) and for each

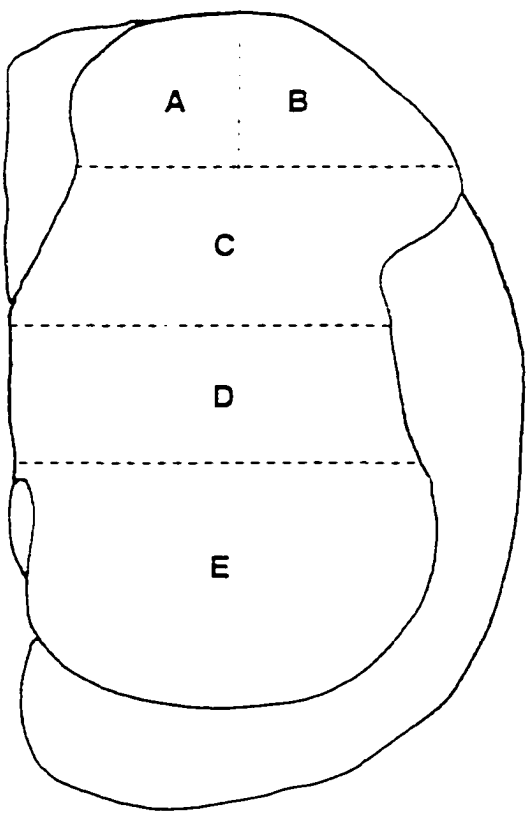
animal the mean number of cell count per region was calculated by averaging the number of stained nuclei from 3 to 5 maximally stained sections. Averaging several sections from each animal increases the power of the ANOVA, therefore requiring fewer subjects for each age and stimulus (Holson and Pearse, 1992). A log transformation was performed on all the data points to correct for heterogeneity of variance and an ANOVA was performed on the total number of stained nuclei as described below. Fisher's Least Significant Difference test (LSD) was used for the post hoc analysis.

The division of the spinal cord. The spinal cord section was divided into 5 different regions. The primary goal of this division was to separate the dorsal and the ventral horn thus distinguishing the sensory (A-D) and motor (E) components of the spinal cord. Secondly, the dorsal horn was further divided to best show clusters of Fos stained nuclei. Areas A and B include superficial laminae where majority of nociceptors terminate, and a large number of Fos labeled neurons are found following nociceptive inputs in the adult. In particular the medial portion of superficial laminae, area A, has shown to express the highest number of Fos immunoreactive neurons following nociceptive signal, consistent with physiological data. The dorsal horn was further divided into C and D. Area C include laminae III and IV which contain cells only responsive to innocuous stimulation. Area D represents deeper layer of the dorsal horn where there are some direct projections from the primary afferent. Therefore, the division used try to follow the physiology of the spinal cord.

Reasons for counting sections with maximum staining. To quantitate cells expressing Fos immunoreactivity, 3 - 5 sections that showed maximum number of staining for each animal were counted. The rationale for this strategy of quantitation was two fold. The most difficult aspect of evaluating immunocytochemical results is to determine what density of label constitutes positive staining. It has been extensively documented that formalin injection into the hindlimb paw pad produces Fos expression that spreads several segments, from upper lumbar to sacral portions of the spinal cord, with a varying degree of staining intensity (Presley et al., 1990). However, the highest number of labeled cells was found at the lumbar enlargement and these cells in addition were most intensely labeled. Therefore, by counting sections that had the most number of stained nuclei, the problem of deciding whether a cell is labeled or not was minimized. Secondly, I was not intersted in quantifying the absolute amount (number) of Fos protein being expressed in the spinal cord following different experimental groups, but in comparing the differences among them. The strategy of cell counting was consistent across all conditions, and therefore, the differences that were observed would indicate the change specifically due to various manipulations. This is particularly true because tissue from different conditions were processed in the same reaction. This method of counting may have provided a more liberal estimate of the number Fos labeled nuclei with a smaller variance than if the entire lumbar sections were counted. Therefore, the changes in the number of labeled cells might have been easier to detect. In addition, if assay conditions for any section are less than optimal, then it is not counted. There are few assay errors that increase label, but many that decrease the staining.

Doses of Morphine The intraperitoneal morphine doses were chosen from the literature and the work previously done in our laboratory (Giordano and Barr, 1984). These doses have been used in the developing animals and they produce significant amount of analgesia. The intracerebroventrical doses were also chosen from the previous work in the laboratory (Barr et al., 1992). The icv doses are much smaller than the ip doses because ICV injection introduces the drug directly into the central nervous system. Although it is possible that morphine administered directly into the brain could have leaked into the spinal cord, the amount of drug injected (10 ug, the highest dose) is not large enough to be analgesic by leakage. To cause significant analgesia when the drug is directly administered into the spinal cord, the doses of the drug have to be between 10 and 30 ug (Barr et al., 1992). Further, the data obtained in this thesis indicate that the leakage of the drug into the spinal cord was not significant. For example, the results indicate that there was consistently greater suppression of Fos immunoreactivity in the lumbar spinal cord compare to the cervical region. If there were leakages of the drug, then the opposite pattern of suppression would have been observed.

Figure 1. Lumbar spinal cord sections were divided into 5 different regions (A-E). Regions A and B include the superficial laminae.



CHAPTER 2:

The Induction of Fos-like Immunoreactivity by Noxious Thermal, Mechanical and Chemical Stimuli in the Lumbar Spinal Cord of Infant Rats

The proto-oncogenes such as c-fos and c-jun play a role as "third messengers" in the transduction of short-term neural events to long-term cellular changes (Goelet et al., 1986). Because Fos can be visualized in individual cells, its assay has been useful in the study of specific neural pathways activated by a variety of stimuli. These stimuli include, among others, generalized seizure (Morgan et al., 1987), dehydration (Sagar et al., 1988), light (Rea, 1989), hypothermia (Joyce and Barr, 1992) and pain (Bullitt, 1989; Menetrey et al., 1989; Bullitt, 1990; Presley et al., 1990; Bullitt et al., 1992; Williams et al., 1990a; Williams et al., 1990b; Bullitt et al., 1992). In the study of pain circuitry, for example, Fos is expressed following stimulation of the hindpaw by noxious thermal (Hunt et al., 1987; Presley et al., 1990), noxious mechanical (Hunt et al., 1987; Bullitt, 1989 and 1990; Bullitt et al., 1992) or noxious chemical stimuli (Hunt et al., 1987; Williams et al., 1990a and 1990b). In each case, c-fos was activated in known primary afferent termination sites: laminae I and II for Ad fibers and laminae I, II and V for C-fibers (Hunt et al., 1987; Presley et al., 1990; Gogas et al., 1991). Furthermore, the topographic pattern of staining, number of Fos stained nuclei and the intensity of the staining are dependent on the intensity and duration of the noxious stimulation (Bullitt, 1989; Bullitt et al., 1992).

The issue of whether infants experience pain has been a controversial and has a long history (Swafford and Allan, 1968; Craig and Grunau, 1993). Recent studies have shown that the requisite pathways for the transduction of painful stimuli develop early in the rat. The primary afferent fibers (Ad and C) enter the dorsal horn L4/L5 segments late on fetal day 19 (FD 19), reach lamina II at FD 19. 5

(Fitzgerald, 1987a) and demonstrate an adult-like distribution through the dorsal horn at birth (Fitzgerald, 1987b). Substance P (SP) and fluoride-resistant acid phosphate (FRAP) are markers for C-fiber afferents (Nagy et al., 1981; Dodd et al., 1984); SP is found in the small primary afferents before birth (Pickel et al., 1982; Fitzgerald and Gibson, 1984), and FRAP is present in the cord within 12 hours of birth (Mattio et al., 1981; Fitzgerald and Gibson, 1984) although adult concentrations of SP and FRAP are not reached until the second week of postnatal life. Electrophysiological properties of fine-diameter nociceptors undergo significant maturation throughout the first week of life (Fitzgerald, 1985; 1988; Fitzgerald et al., 1987). Thus, although the nociceptive pathways are present early, they continue to develop throughout early postnatal life.

The behavioral data seem to be consistent with development of the electrophysiological and neurochemical properties of these fine-diameter nociceptors: pups are capable of responding to noxious stimulation at or before birth but the adult behavioral pattern to stimulation continues to develop well into postnatal life. The flexor-withdrawal reflex is present in response to pinching and heating the hindpaw on the day of birth (Fitzgerald and Gibson, 1987) and dorsal horn cells are responsive to pinching of the distal hindlimb at FD 19 (Fitzgerald, 1991). The capsaicin-sensitive burst of spikes elicited by electrical stimulation, however, does not appear in these cells until postnatal day 9 (PD 9) (Fitzgerald and Gibson, 1984). The use of other functional markers has provided additional information about nociceptive processing during development. Williams et al. (1990a) reported that formalin injected into the plantar pad of the hindpaw or subcutaneous injection of capsaicin induced the Fos protein a small number of

dorsal horn neurons in 1 day old anesthetized rat pups. In contrast, the application of mustard oil to the hindpaw produced no Fos-labeled nuclei in 3 day old rats, the oldest age tested.

The aim of the present study was to examine the maturation of second-order neurons in response to stimulation of fine-diameter nociceptors in the preweanling rat using the expression of c-fos as an anatomical and functional marker with mechanical, thermal and formalin as noxious stimuli.

METHODS

Peripheral Stimulation

Formalin. Zero-day old (n=3) animals were injected with 10 µl of 10% formalin; 1 (n=6) and 2 day old (n=5) pups received 10 or 15 µl of 10% formalin; 3 day old (n=9) animals received 10, 15 or 20 µl; and the 14 day-old (n=3) pups were injected with 20 µl of 10% formalin. Formalin was administered subcutaneously in the plantar surface of the right hindpaw.These volumes were derived from comparable studies in the adult animal, prior developmental work and from pilot studies. The one prior developmental study utilized 10 µli of 4 % formalin in 1 to 3 day old pups (Williams et al., 1990a). The volumes used in the adult have ranged from 50 to 150 µl (Abbott and Melzack, 1982; Dickenson and Sullivan, 1987; Noguchi et al., 1989; Presley et al., 1990; Williams et al., 1990b; Gogas et al., 1991; Abbadie et al., 1992; Coderre et al., 1993). The volumes used here were decreased of necessity because of the smaller size of the paw and were consistent with the Williams et al., (1990b) study, although we used a higher concentration of

formalin. Control animals were injected with an equal volume of saline also in the right hindpaw. All animals were perfused 2 hours post-injection.

Thermal. Initially, animals were allowed to withdraw their paw from a hot water bath 52 ° C. They withdrew their paws instantly and this protocol produced no or very few Fos stained nuclei in the spinal cord. Thereafter, the paw of 0 or 1 (n=4), 2 (n=2), 3 (n=2) and 14 day old (n=2) pups were held submerged in the water bath for 5 seconds at 5 minute intervals for 15 minutes. The hindpaw of control animal was immersed in a neutral temperature water bath (32 ° C) for the equivalent time period. The animals were perfused 2 hours after the end of the stimulation.

Mechanical. The right hindpaws of 0 (n=2), 1 (n=3), 2 (n=2) and 3 day old pups (n=3) were pinched with a small arterial clip for 2 seconds (5 seconds for 14 day old pups, n=2), 4 times every 5 minutes for 15 minutes. For the control animals, right hindpaw was gently stroked with a paint brush for the equivalent time period. The animals were perfused 2 hours after termination of stimulation.

Twelve immunocytochemical reactions were performed in this experiment. Although day-to-day variability in the assay was small, for each set of reactions at least 1 control animal was processed with the experimental animals and at least two different age groups were processed togther. Except for these contraints tissue was assayed in a non-systematic manner to avoid biasing the data dut to day-to-day assay variability. To determine more directly if there were systematic differences on different assay days, a one-factor analysis of variance (ANOVA) wa conducted on the number of stained nuclei assayed each day. Post-hoc tests showed that 1 assay day had significantly more stained nuclei than did the 5 days with the lowest

number of stained nuclei. there were no other significant differences. The 1 assays that differed contained proportionally greater numbers of animals treated with the highest formalin volume than did the other assays. Nonetheless, even within this assay, the number of labeled nuclei varied from 79 to 419, well within the range of the other assay days. Thus the number of stained nuclei reported here is most likely due to the experimental maninpulation and not an artifact of the immunocytochemical reaction protocol.

RESULTS

General behavioral observation

Immediately following injection, swelling of the injected paw was observed in animals of all ages and at all injection volumes for the formalin group. The edema lasted approximately 60 minutes. The most common behaviors exhibited were vocalization during injection and paw lifting following injection. Paw licking was observed in very few animals. For the saline injected animals, vocalization was infrequent and edema of the injected paw was observed only in a few animals; typically the duration of edema was much shorter than the for formalin treated groups. Animals treated with either the mechanical or the thermal stimulation showed mild edema of the injured paw in all ages. Paw licking was not seen but paw lifting and vocalization during the administration of the noxious stimulation was observed frequently.

These was no basal Fos staining. Staining occurred only following stimulation. The immunoreactive product was localized exclusively to the nuclei. Injection of 20 µl of saline resulted in some staining in the superficial laminae but control groups receiving less than 20 µl of saline demonstrated no staining. Few, if any, Fos-positive neurons were apparent on the unstimulated contralateral side. In a few animals, faintly stained Fos nuclei were seen contralaterally, but in no case was staining abundant.

Formalin

At all ages and for all formalin injection volumes, the medial portion of the superficial laminae had the highest number of stained nuclei, a result consistent with anatomical projection of nociceptive primary afferents from the paw(Fig. 2).

Injection of 10 µl formalin in 0, 1, and 2 day-old pups resulted in lightly labeled nuclei mainly in the superficial laminae. Injection of the 3 day old animal with 10 µl of formalin resulted in a dramatic increase in the number of Fos stained nuclei (Fig. 3). A one way ANOVA on the 0 to 3 day old age groups that received 10 µl of formalin showed a statistically significant increase in the number of labeled neurons, $F(3, 8)=4.23$, $p< 0.05$. Fisher's LSD post-hoc analysis revealed that the 3 day old animals had significantly more stainde nuclei than did the other age groups, which did not differ from each other.

To assess whether increased afferent input would increase the number of labeled nuclei, different volumes of formalin were injected. An ANOVA of the volume (10 and 15 µl) and age (1, 2 and 3 days of age) showed that the animals treated with the

Figure 2. The mean number (± SEM) of fos immunoreactive nuclei in response to 10 µl (20 µl for 14-day-old pups) of 10 % formalin found in different regions of the cord (see Fig. 1). Total represents the sum of the labeled nuclei for each age.

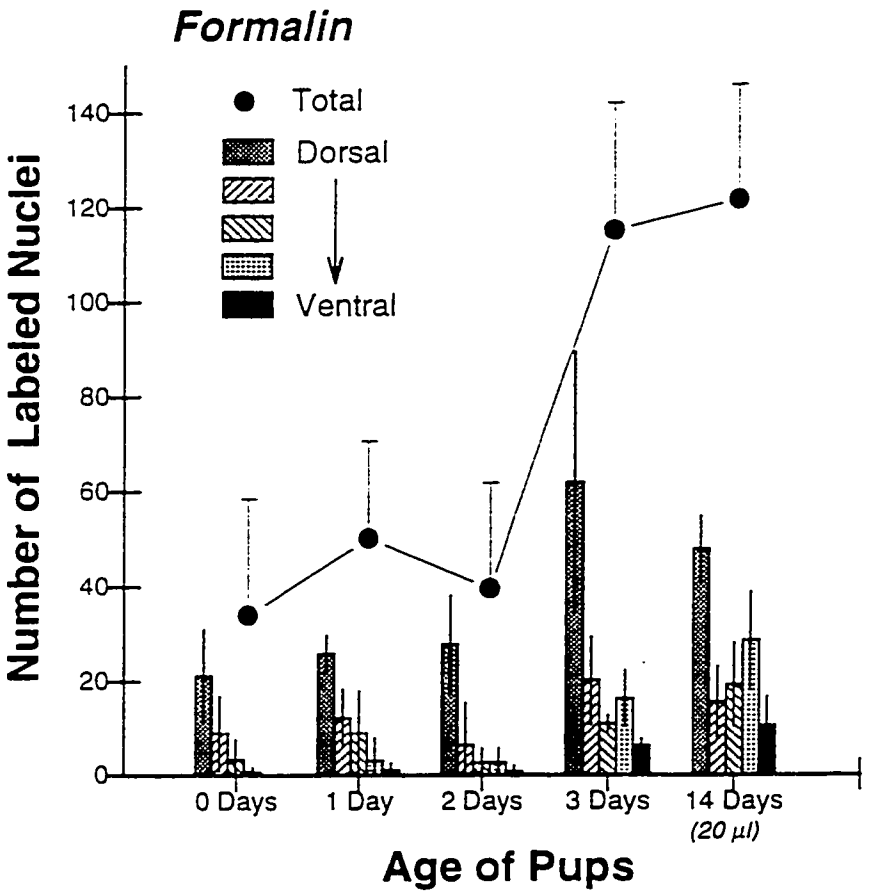
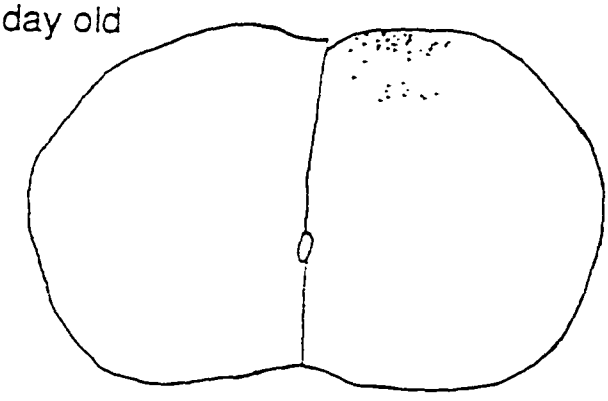


Figure 3. Camera lucida drawings of Fos stained nuclei in the dorsal horn of the spinal cord induced by 10 µl of 10 % formalin for 1 day old and 3 day old pups. The 3 day old groups shows a greater number of stained nuclei than does the 1 day old animal.

1 day old



3 day old

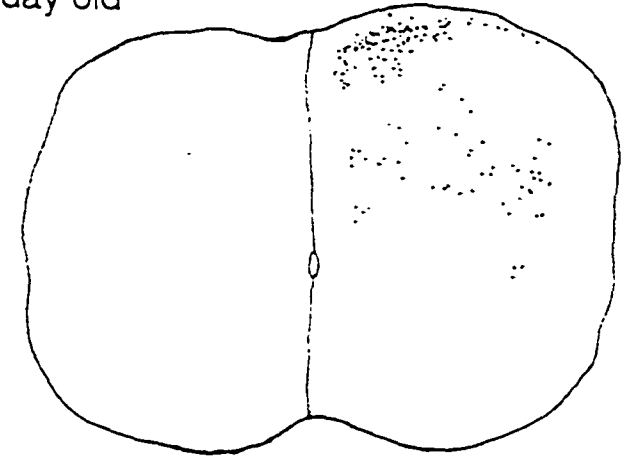


Figure 4. The mean number (± SEM) of Fos-stained nuclei following 10, 15, 20 µl of 10 % formalin in 3-day-old pups. Details are the same as for Fig. 2.

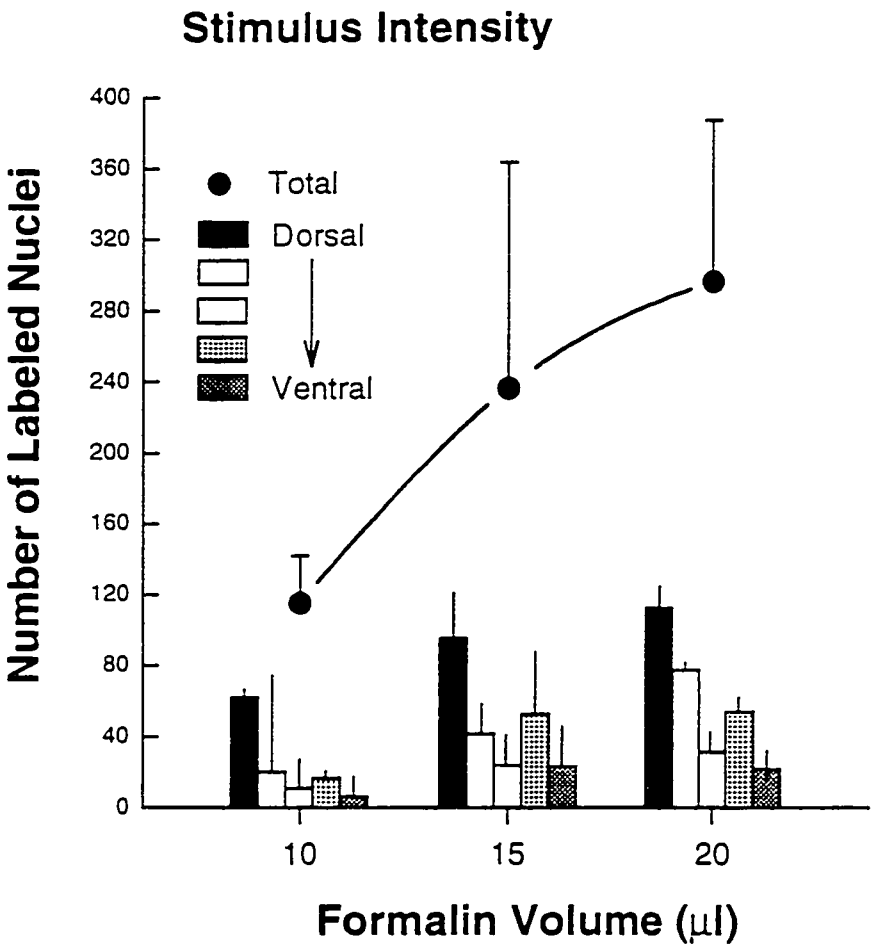


Figure 5. Fos expression was studied with different formalin injection volumes. A: photomontage of the 3 -day-old lumbar spinal cord following injection of 10 µl of 10 % formalin. B: photomontage of labeled nuclei in the dorsal horn and ventral horns of the lumbar spinal cord of the 3-day-old following injeciton of 20 µl of 10 % formalin. Staining is apparent throughout the dorsal horn and at this volume spreads into the ventral horn. More Fos-labeled neurons are seen in the animals treated with 20 µl volumes thatn with 10 µl. Bar: 50 µm.

15 µl of formalin resulted in a greater number of Fos stained nuclei than did the 10 ml group, $F(1, 12)=8.126$, $p< 0.05$. This was true both at 2 and 3 days of age, although the number of labeled nuclei was greater in the older animals for the two volumes. To study this further, we injected 3 day old pups with 10, 15, or 20 µl of formalin. The mean number of stained nuclei for 15 and 20 µl was similar (236 and 296, respectively) (Fig. 4) and these two groups were compared together to the 10 µl injection group. The animals treated with the higher volumes of formalin had significantly more Fos-positive cells than did animals receiving the lower volume, $F(1, 7)=7.09$, $p< 0.05$. These data are shown graphically in Fig.4. Figure 5 presents photomontages of stained transverse sections for 10 µl and 20 µl injection volumes, and illustrate that with the greater injection volume, more nuclei are labeled and the staining is apparent in the ventral horn of the spinal cord as well.

Mechanical

All the staining observed with the mechanical stimulation was in the superficial laminae with an exception of the 0 day old pups. Newborn pups treated with mechanical stimulus, in addition to the large numbers of lightly stained Fos immunoreactive nuclei in the superficial laminae, had a limited number of labeled nuclei in deeper layers (Figs. 6 and 7). The number of Fos stained nuclei from 0 to 14 day old animals was compared. The 0 day old pups expressed more Fos positive neurons than did the 3 day old pups, $F(4, 7)=7.37$, $p<0.05$. Fisher's LSD post hoc analysis revealed that the 0 day old pups were statistically different from 1, 2, and 3 day old pups ($p<.05$). The 14 day old pups differed only from the 2 day old pups.

Figure 6. The numean number (± SEM) of Fos-labeled nuclei following mechanical pinch in 0-. 1-. 2-, 3- and 14-day-old pups. Details are the same as for Fig. 2.

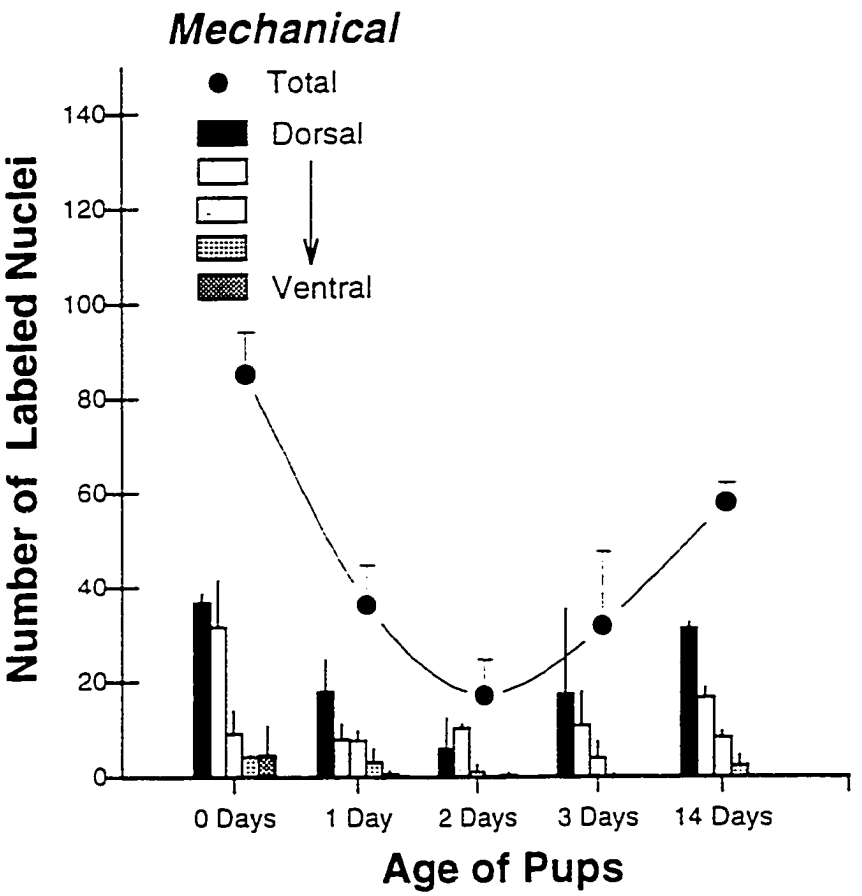


Figure 7. Camera lucida drawings of Fos labeling following mechanical stimulation for 0 day old (1 and 2 day old pups were not substantially different from the 0 day old animals), 3 day old and 14 day old pups. There were more Fos-labeled nuclei in the 0 than in the 3 day old pup.

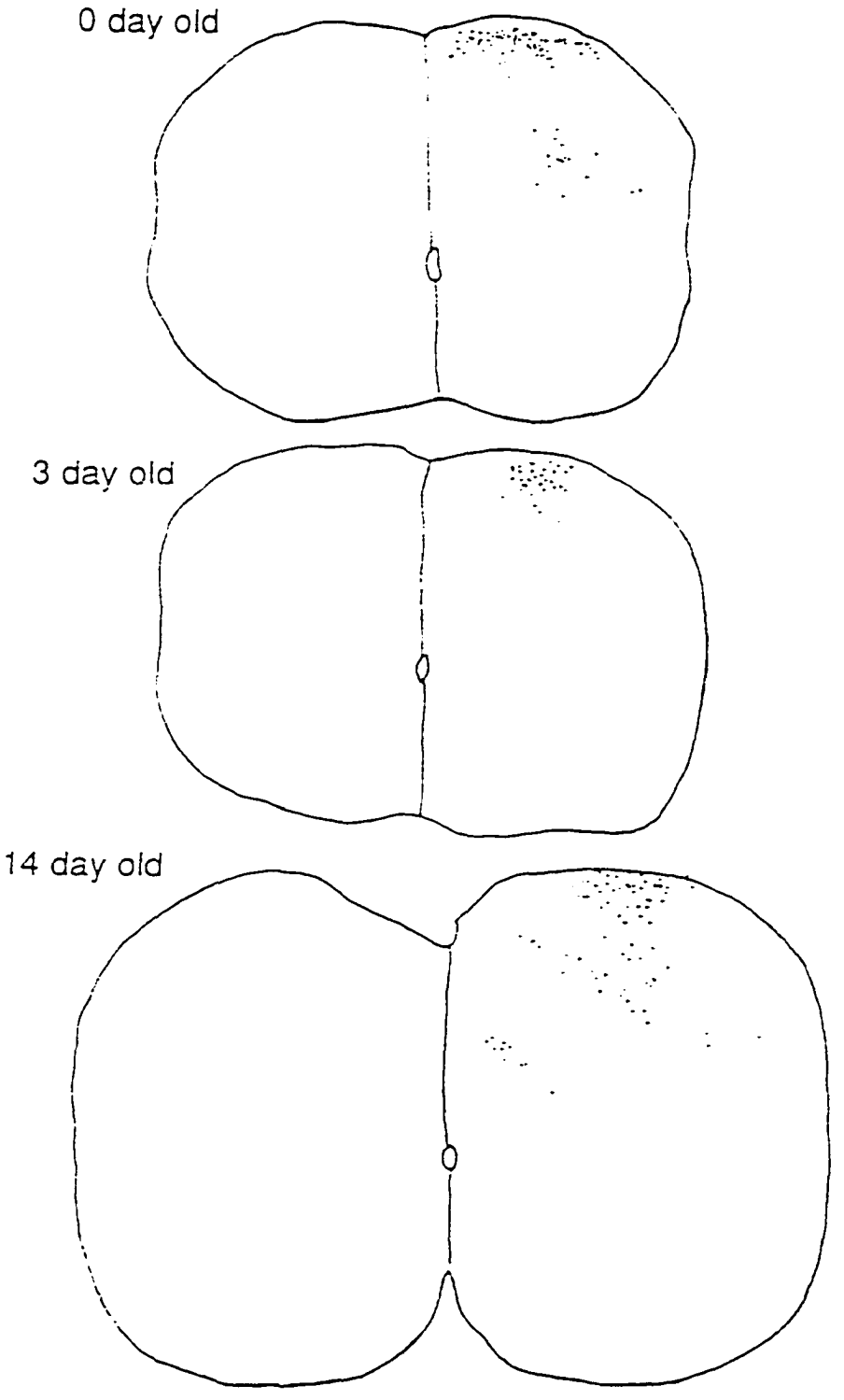


Figure 8. The mean number (± SEM) of stained nuclei induced by hot wat in 0 . 1 . 2. 3and 14 day old animals. Details are the same as for Fig. 2.

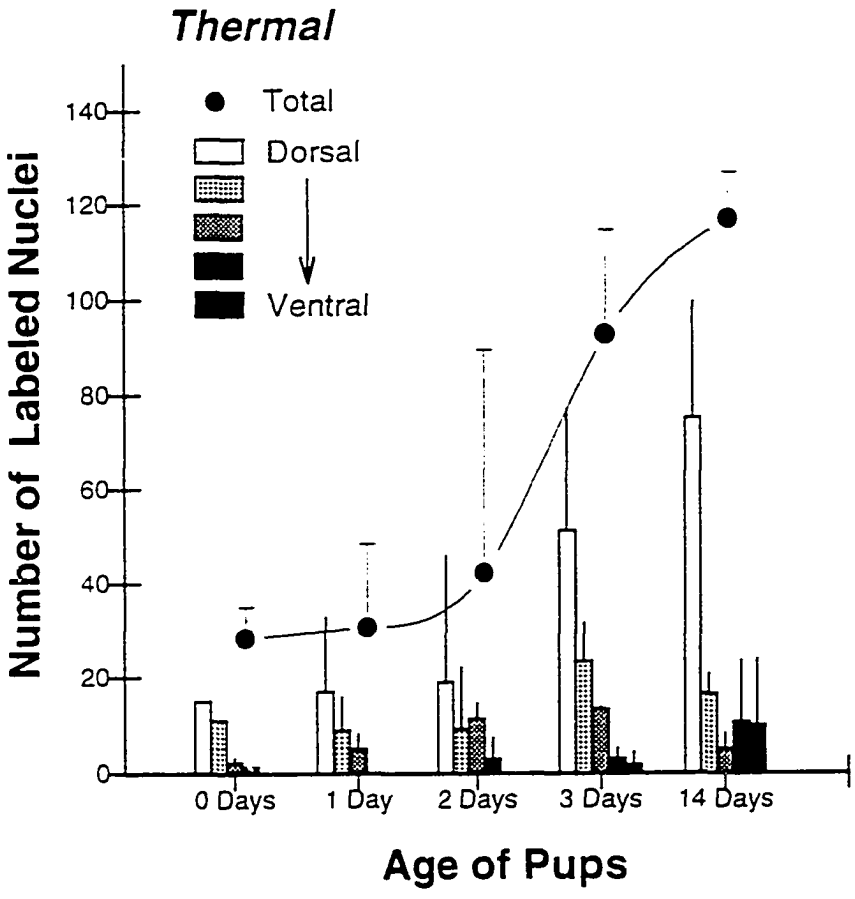
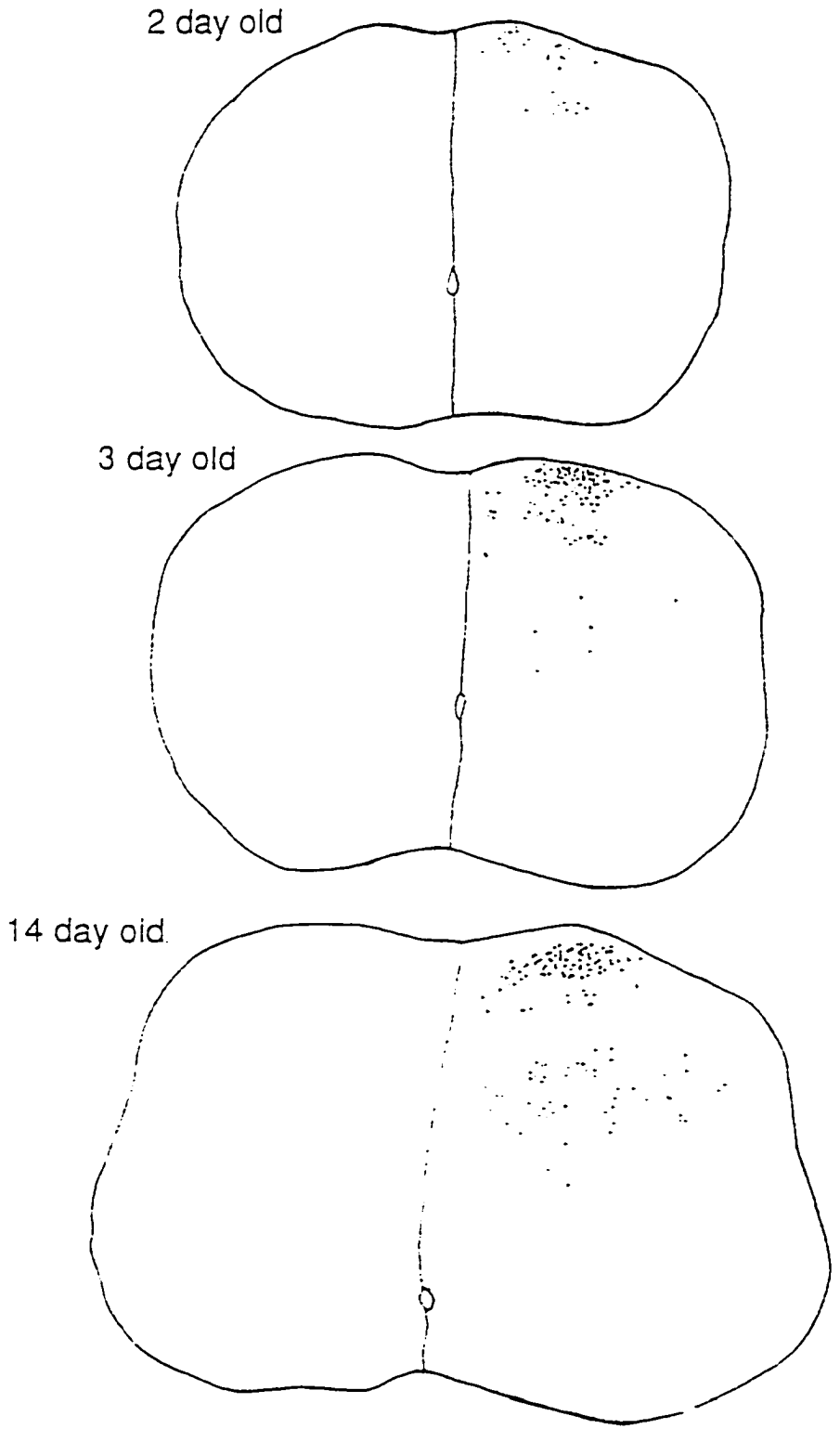


Figure 9. Camera lucida drawings of lumbar spinal cord sections of animals showing the Fos-stained nuclei after their hindpaws were immersed in hot water. The 0 and 1 day old groups were similar to the 2 day old animals.



Thermal

Examination of data from animals treated with the noxious thermal stimulus showed that the pattern of Fos expression and number of Fos labeled nuclei changed as a function of age. In the younger age groups (0 to 2 day old). there were relatively few Fos immunoreactive neurons and these occurred largely in the superficial laminae; in older pups there were greater numbers of labeled cells and progressively more ventral horn neurons were stained (Figs. 8 and 9). Statistical analysis showed that significantly more stained cells was seen in the older age groups (3 and 14 day old animals) than in the younger age groups (0 to 2 day old pups). $F(2, 8)=7.39$, $p<0.05$.

DISCUSSION

The results of this study show that expression of the Fos oncoprotein can be evoked at any postnatal age including the day of birth. by three different peripherally applied noxious stimuli. Our results are consistent with those of Williams et al. (1990a) who showed that the Fos immunoreactive cells were observed in 1 day old animals. Other studies have shown that fine-diameter C-fiber are not fully mature until the second week of postnatal life for the rat when measured electrophysiologically (Fitzgerald et al., 1987b; Fitzgerald, 1988). The appearance of the Fos protein induced by noxious sensory stimulation indicates that nociceptive information can be transmitted to the second order neurons at birth. The nociceptive signal is enhanced by further maturation as evidenced by the increased expression of the Fos protein in the older pups in response to the thermal and

chemical stimuli. This result is consistent with the electrophysiological and anatomical data.

Although the number of subjects at each data point was small, we chose to present the data for each age separately rather than combing certain ages. The pattern of results proved developmentally consistent. For example, for both thermal and formalin stimulation, the number of labeled cells was low and constant for the 0 to 2 day old pups. For both stimuli the number of labeled nuclei increased at 3 days of age. Likewise, increasing volumes of formalin resulted in more labeled nuclei at all ages tested. The pattern of results for the mechanical stimulus differed. Although we did not systematically record the behavioral responses of pups to this stimulus, our impression was the the pressure clip resulted in a more vigorous response in the newborns than in the older pups which might account for the greater number of stained nuclei in the neonates. This obsrvation requires further systematic study to determine whether the developmental U-shaped function (Fig. 6) is a unique result of the mechanical stimulus or is due to a more intensely noxious signal "perceived" by the 0 day old pups.

Our experimental manipulation (e.g., increased formalin volume) resulted in increased expression of the Fos protein even in the 1 day old pups, suggesting that the system is able to respond to an increasingly noxious stimulus at an early age. For example, although the number of stained nuclei in thermally trated 0 to 2 day old animals was only one-half that of the 3 day old pups, it is probably not a limitation of the c-fos gene because 15 µl injections of formalin in 1 and 2 day old

animals resulted in approximately the same number of labeled celles as was seen in the 3 day old pups exposed to hot water.

For animals treated with either 10 µl of formalin or the noxious thermal stimulus, the 3 day old age group had significantly more labeled nuclei than did the younger age groups. It is likely, therefore, that at this age there are maturational changes in some aspect of the synaptic connections between the primary afferents and the second-order dorsal horn cells. According the electrophysiological dat, dorsal horn cells respond to electrical skin stimulation at PD 9 (Fitzgerald, 1988) and the flexion reflex produced by capsaicin treatment is not seen until PD 10 (Fitzgerald and Gibson, 1984). These data suggest continued maturation of the processing of noxious stimuli during the 2 weeks of life. Our results are consistent with these electrophysiological data, although we did not determine whether maturation continues to increase between 3 and 14 days of age.

Comparison of data from the different aged animals treated with 10 µl of formalin showed that spinal cord neurons in older pups expressed the Fos protein more in ventral horn neurons than did younger animals. In 0 day old pups, all of the Fos stained nuclei were confined to the dorsal horn (regions A-C in Fig.1) where the nociceptive primary afferents mediating pain terminate. By 3 days of age, however, 20% of the immunoreactive neurons were found in the ventral horn. It was recently reported that in adult rats injeciton of formalin in the hindpaw resulted in a laminar distribution of fos immunoreactive neurons that also included the ventral horn (Presley et al., 1990; Williams et al., 1990b; Bullitt et al., 1992). Fos positive ventral horn cells are polysynaptically activated by cells in the superficial dorsal

horn laminae (Williams et al., 1990b) and are more profoundly suppressed by systemic administration of morphine than are the immunoreactive neurons of the dorsal horn (Presely et al., 1990). In the infant rat, as it matures, Fos immunoreactive nuclei in ventral horn cellls also become involved in the processing of noxious stimuli. Whether they are differentially responsive to analgesic drugs remains to be tested (Presley et al., 1990).

Recently, the influence of stimulaiton duration on fos staining in adult rat dorsal horn cells was addressed directly (Bullitt et al., 1992). The topography of Fos labeled nuclei in the dorsal horn was a function of the duration of noxious stimulation to which the animals were exposed. Brief stimulaiton evoked faintly labeled nuclei mainly in the superficial laminae wheras continuous or long stimulus durations produced more densely labeled nuclei both in the superficial and ventral laminae of the cord. In our results, stimulus intensity influenced both the number of immunoreactive neurons and intensity of staining.These effects of stimulus intensity were most directly demonstrated with formalin treated animals. In the 1 to 3 day old pups, increasing the injection volume from 10 to 15 µl increased the number of stained nuclei; incrementing the volumes from 15 to 20 µl in the 3 day old group did not further increase the number or intensity of stained nuclei. Whether this is because the maximun number of expressed c-fos staining in the 3 day old is developmentally limited or whether 20 µl is not significantly more "painful" than 15 µl is not known. Increased staining induced by the increased volume could be due to a longer duration of noxious stimulation, to stimulation of more dermatomes, to increased irritation or to some combination of the above factors.

There is further evidence in the thermally stimulated animals that stimulus duration exerts an effect on c-fos expression, although these data are less direct. Less intense thermal stimulaiton (i.e., allowing the pups to withdraw their paws) failed to induce the c-fos gene whereas longer duration stimulation (5 seconds) did. this confirms that stimulus duration in the infant as well as the adult is an important component in the induction of Fos immunoreactivity for a thermal noxious stimulus. Therefore, as in the adult, expression of the Fos protein is directly proportional to stimulus intensity and duration in the very young rat pup.

In summary, nociceptors are functional early in postantal life and the topographic pattern of termination on second-order neurons is similar to that of adult animals. Fos like immunoreactive neurons are found in the superficial and deeper layers of the dorsal horn and in the ventral horn neurons. These results confirm the hypothesis that the pain system develops early in the rat but that spect of the affernt input mature during the first week of postantal life.

CHAPTER 3:

The Expression of *C-fos* Gene in Response to Formalin Injection in the Spinal Cord of Prenatal Rats

INTRODUCTION

The basic connections in nociceptive pathways are present before birth both in human and rat fetuses. For example, neurons in the spinal cord are generated between gestation day (GD) 11 and 16, with cells in the substantia gelatinosa appearing during the final stages of spinal cord neurogenesis between GD 14 and 16 (Nornes and Das, 1974). The interneurons in the substantia gelatinosa initiate their axonal and dendritic maturation just before birth, GD 21, and continue to mature well into postnatal life (Bicknell and Beal, 1984; Fitzgerald et al., 1991). The nociceptive primary afferents, Aδ and C-fibers, are found in the dorsal horn at GD 19.5- 20 (Fitzgerald, 1987a), but an adult-like C-fiber terminals are observed only after postnatal day (PD) 5 (Pignatelli et al., 1985). The functional maturity of the system corresponds with the anatomical data. For example, fetal primary afferents respond to noxious inputs starting at GD 17, but the dorsal horn cells do not respond to these inputs until GD 19 with a normal dorsal horn neuronal activity occurring in the second week of rat's postnatal life (Fitzgerald, 1987). Behavioral data are also consistent with the anatomical and physiological maturation of the nociceptors and the dorsal horn cells. Forelimb cutaneous reflexes are evoked on GD 17 (Narayanan, Fox, and Hamburger, 1971), and by birth flexor withdrawal reflexes to pinching and heating the skin are present (Fitzgerald and Gibson, 1987). The adult pattern of response is observed with increasing postnatal age (Fitzgerald et al., 1988; Fitzgerald and Gibson, 1987).

In the past decade the expression of proto-oncogenes such as C-*fos* has been extensively used as a tool to mark activated neurons in the central nervous system.

C-*fos* expression is induced by a variety of stimuli such as seizures (Morgan, Cohen, Hempstead and Curran, 1987), hypothermia (Joyce and Barr, 1990) and pain (Abbadie et al., 1992; Hunt et al., 1987; Menetrey et al., 1989; Presley et al., 1990; Williams et al., 1990a; Yi and Barr, 1995). In examining mechanisms of pain processing particularly at the spinal cord level, use of the C-*fos* gene expression as an anatomical marker has been especially powerful and many studies have demonstrated Fos-positive nuclei in the known termination sites of the nociceptive inputs in the spinal cord of both neonatal (Williams, et al., 1990b; Yi and Barr, 1995) and adult rats (Presley et al., 1990). There is also evidence that target genes for C-*fos* may be the endogenous opioid genes, preproenkephalin and preprodynorphin (Noguchi et al., 1991; 1992), consistent with the putative role of C-*fos* gene in pain circuitry. Neonatal administration of capsaicin, which selectively destroys the small unmyelinated primary afferent, reduces the levels of Fos-like-immunoreactivity and preprodynorphin mRNA further implicating C-*fos* proto-oncogene in the regulation of dynorphin gene transcription (Hylden et al.,1992).

Following application of formalin, noxious mechanical or noxious thermal stimuli, the C-*fos* oncogene is expressed in the spinal cord as early as on the day of birth (Yi and Barr, 1995). The presence of the Fos protein following peripheral stimulation suggests that the primary afferents are functional and the dorsal horn cells, although immature, are capable of turning on C-*fos* gene in response to these peripheral stimuli. The goals of present study were to examine 1) fetal behaviors following treatment with formalin in the paw, 2) the expression of C-*fos* gene in the spinal cord of GD 19, 20, and 21, and 3) the maturational difference between

cervical and lumbar spinal cord neurons by injecting either the forepaw or the hindpaw.

METHODS

Subjects

An adult female rat was placed with a male rat for 14 - 16 hours overnight. On the following day, a vaginal smear was taken and with the presence of sperm, that day was designated as GD 0. Subjects were 10 litters of GD 19 (3 litters), GD 20 (4 litters), and GD 21 (3 litters) fetal rats obtained from time mated dams in our animal facility. Litter sizes averaged 10 fetuses, but approximately 4 to 6 were tested to increase the survival rate of the fetuses, because there was a positive correlation between the number of fetuses injected (tested) and the likelihood of placental detachment.

In utero preparation.

On GD 19, 20, or 21 the dam underwent chemomyelotomy at L1/L2. Chemomyelotomy was performed by injecting 80 - 100 µl of 100% ethanol directly into the spinal cord at L1/L2. This procedure paralyzed the dam and as a result the dam was awake, but had no sensory or motor functions caudal to the site of the ethanol injection (Smotherman & Robinson, 1991). Dams were not anesthetized because anesthetics cross the placenta and the fetuses would be exposed to them. Anesthesia has shown to suppress the expression of *C-fos* gene in the adult (Dragunow and Faull, 1989) and in neonates (Yi and Barr, 1996). The dam was placed in a holding device and her caudal half submerged in a warm bath containing Locke's solution. The temperature of the bath was carefully monitored and

maintained at 37.5 ± 0.5 °C. An abdominal incision was made and the uterine horns were externalized into the bath by applying pressure around the incision site. The dam and fetuses were allowed to acclimate for 15 - 20 minutes. A fetus was chosen at random and a small cut was made in the uterine wall to expose the chosen fetus. The amniotic sac and its surrounding membranes were carefully peeled away from the fetus, leaving it attached to its placenta via the umbilical cord. The fetus was then injected unilaterally with 5 µl of 10% formalin or saline into either the forepaw or hindpaw. The dam and the fetuses were left undisturbed for 2 hours at which time fetuses were removed from the dam and overdosed with a barbiturate.

Fetal Behavioral Observation

Following the injection of a paw, a fetus was observed continuously for 5 minutes. Any and all behaviors exhibited by the treated fetus was recorded. Only one fetus was observed at a time. The fetal observation time was limited to 5 minutes due to a concern over the quality of the preparation. The longer the fetuses were *ex utero*, the weaker the fetuses might have become and therefore would have affected the Fos immunoreactivity although we have not seen any evidence of this for this experiment.

Quantiation

The sections were counted using a division shown in Fig. 10. Fos positive cells found in the most superficial layer and Fos labeled nuclei found in the remaining areas were separately counted. Ipsilaterally and contralaterally stained cells were counted and recorded separately. There was bilateral staining in the deeper layers of

dorsal horn in untreated, saline and formalin injected animals, suggesting that the observed bilateral staining was mainly a developmental effect, not an effect due to the independent variable, the formalin injection. Therefore to assess the contribution of the formalin injection, the cell counts were transformed into a difference score by subtracting Fos labeled nuclei found contralaterally from ipsilaterally stained cells. Data were first analyzed using transformed cell counts. In the subsequent analysis, to examine the difference between ipsilaterally and contralaterally stained cells, original data, the non-transformed cell counts were used. A factorial analysis of variance (ANOVA) was performed on the number of cells stained with Fos protein. The Newman-Keuls test was used for post-hoc comparisons.

RESULTS

Behavior

Fetal behavior following the formalin injection changed with increased fetal age. The oldest fetus (GD 21) showed well coordinated behaviors that were not part of their normal behavioral repertoire. The behavior following formalin treatment included body curls (curling the entire body), body twitches (a short- lasting jerky movements), fore or rear leg movement, mouth opening, and face wiping (swiping of the sides of the face with two forepaws). Behaviors also occurred that were specific to either forepaw or hindpaw injections. Face wiping was seen only following the forepaw, and not hindpaw formalin injection. The hindpaw injection caused the animal to flex its injected limb, resembling postnatal animal's paw lifting (Guy and Abbott, 1992). GD 20 fetuses showed less complex behavior following formalin injection than did the GD 21 animals. For example, only body curls and

body twitches, and movement in the injected paw were observed following the formalin treatment. GD 19 fetuses showed even fewer responses following the formalin injection. Although one litter of the GD 19 fetuses showed mouth opening and the movement in the injected paw, most fetuses demonstrated only a slight increase in the general body movement. No specific injury directed behavior was observed. None of the fetuses at the 3 age groups tested here showed any behavior following saline injection with an exception of a short-lived increase in activity that occurred immediately following the injection. Formalin injection produced redness and swelling in the injected paw in all animals, including GD 19 fetuses, and there was a little difference in the degree of edema observed among the 3 age groups. Saline injection did not produce any edema.

Anatomy

As shown in Figure 10, clusters of faintly stained cells were consistently found bilaterally in lamina III across all ages and conditions (untreated, saline and formalin conditions), both in cervical and lumbar cord (Fig. 11). In stark contrast to the formalin-induced labeling that was intensely stained, the constitutive labeling was always faintly stained. In addition, the intensity of formalin-induced labeling was age dependent (Fig. 12). GD 20 and to some extent, GD 19 fetuses treated with formalin produced darkly stained nuclei mainly in the superficial layers, with the distinctive clusters of faintly stained cells in deeper layers. By GD 21, fetuses injected with formalin showed darkly stained nuclei in the superficial lamina and also deeper layers of the dorsal horn, along with the clusters of faintly stained

Figure 10. Photomontage of the lumbar spinal cord of the GD 21 untreated fetus. Faintly stained Fos nuclei are observed bilaterally and was consistently observed for all gestational ages and at the cervical and lumbar segments of the spinal cord.

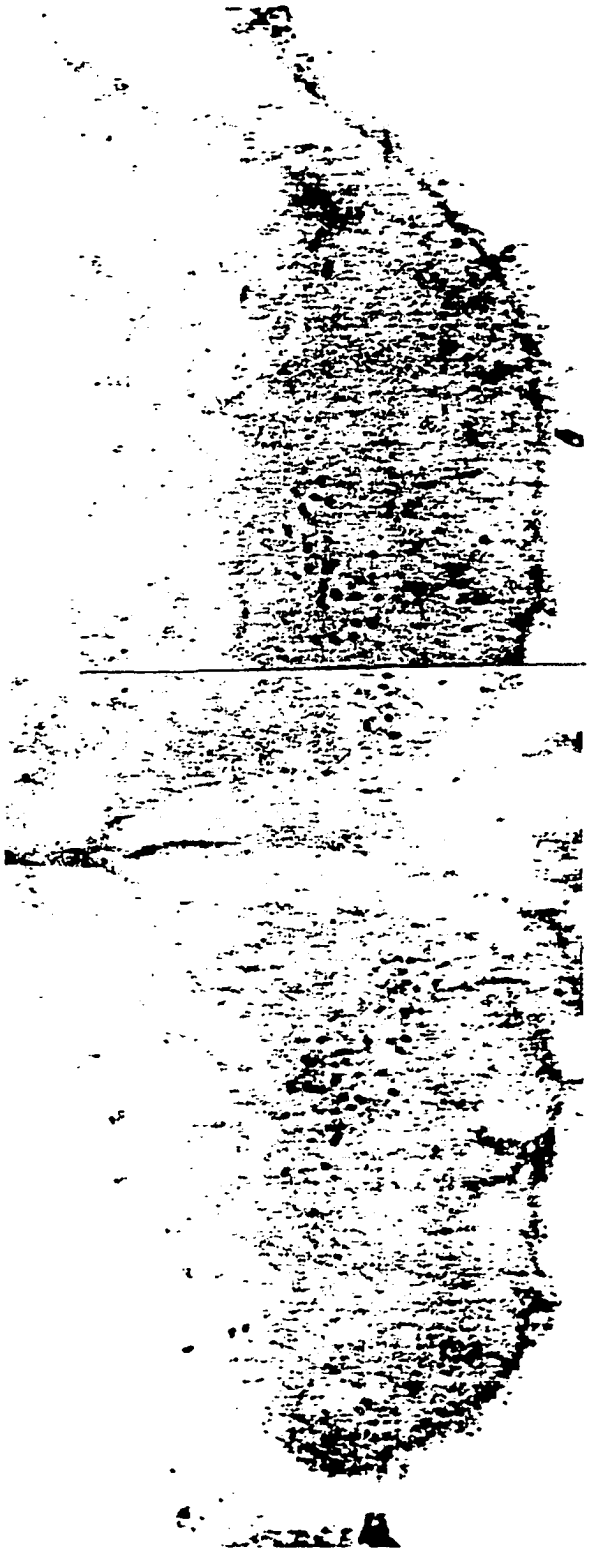


Figure 11. Camera lucida drawings of Fos labeled nuclei in the dorsal horn of the spinal cord following formalin injection in GD 19. 20. and 21 fetus at both cervical and lumbar spinal cord. Labeled nuclei are apparent both on the stimulated (right half) and in the non stimulated side (left half).

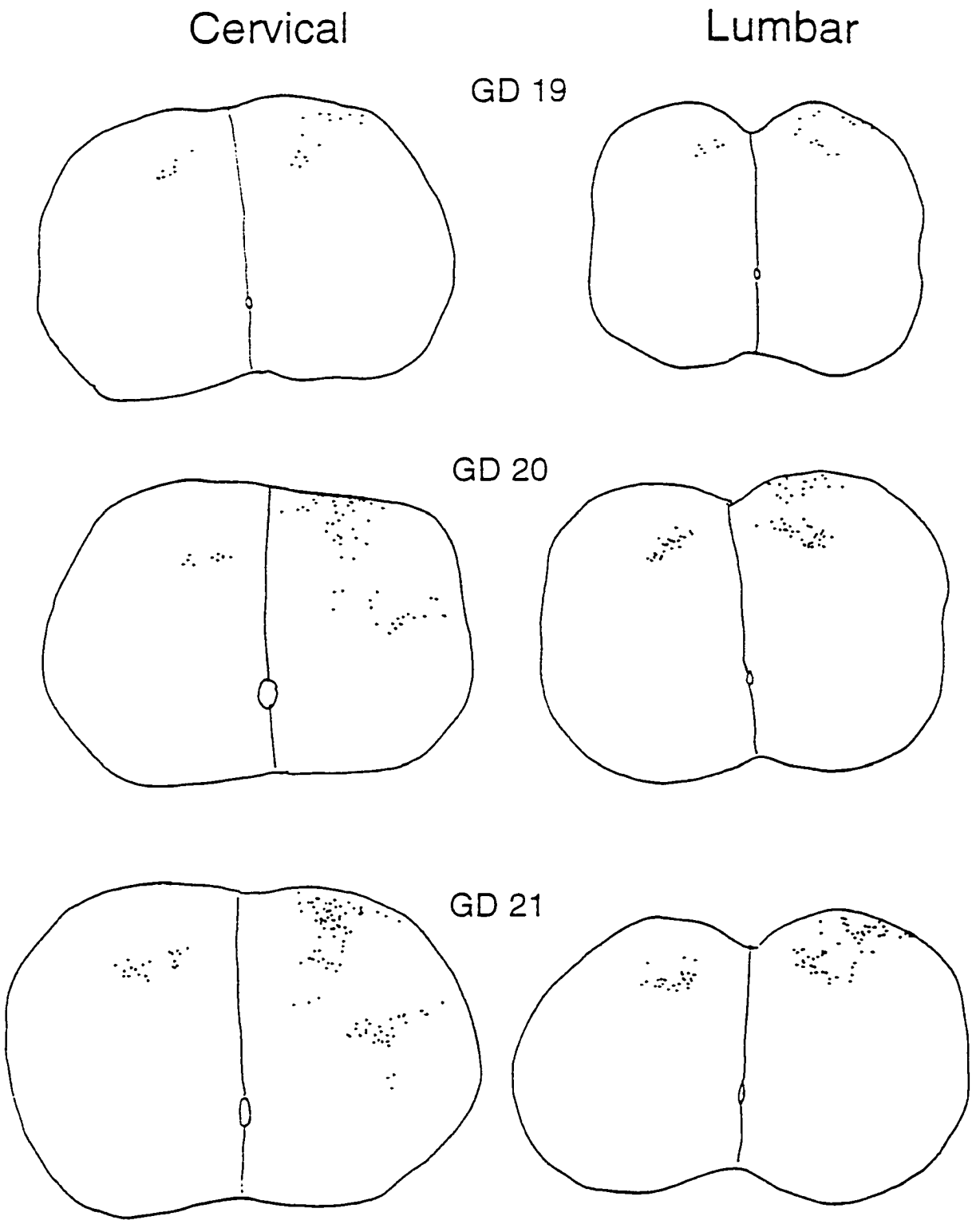


Figure 12. Photomontage of the lumbar spinal cord of GD 19 fetus injected with 5 μl of formalin. Only few fos labeled cells are observed. The stimulated side (right) has few Fos stained cells in the very superficial portion of the dorsal horn.

nuclei in lamina III. Uninjected fetuses showed lightly stained Fos cells that were similar to those seen in saline injected animals.

Development. As shown in Figures 13 and 14, formalin increased the number of Fos-like-immunoreactive (FLI) cells across age, (treatment x age), $F(2, 7) = 9.58$, $p=.0099$, with the largest increase occurring between GD 20 and 21, suggesting that as the fetus matures there is an increase in the number of Fos stained nuclei following the formalin injection. This increase was not seen in the saline treated animals. Figure 14 shows that most of the increase in the number of Fos positive cells was due to an increase in the most superficial layers of the cord, and not due to an increase in the deeper layers, (treatment x lamina x age), $F(2,7) = 9.035$, $p=.0115$. This effect was consistent for all ages. In the deeper layers, there was only a trend for formalin to increase Fos expression in an age dependent manner. In saline treated animals, Fos stained cells were seen only in the deeper layer, and this effect did not change with age, suggesting that the constitutive level of Fos staining remains constant across the 3 fetal ages tested here.

Ipsilateral vs. contralateral staining. Figure 15 shows that there was a greater number of Fos stained cells following formalin treatment in the ipsilateral side compared to the side contralateral to the injected paw. This effect was age dependent. The number of Fos stained nuclei in the contralateral side were similar for the saline and formalin treatments for all the age groups, suggesting that formalin did not induce contralateral Fos expression at any age. No superficial Fos-positive cells were apparent in the ipsilateral or contralateral cord following a saline injection (Fig. 1 5). In addition, there was no difference between saline and

Figure 13. Photomontage of the lumbar cord of GD 21 animal injected with 5 µl of formalin. Note both the increase in the number of labeled nuclei and the intensity of the staining compared to the GD 19 fetus seen in Fig.12. Injection was ipsilateral to the right side of the spinal cord. The dashed line indicated the demarcation of "superficial" and "deeper " layers.

Figure 14. Histograms of the means number of Fos labeled nuclei found at GD 19, 20, and 21 following either formalin or saline injection. The data are transformed difference scores, and the number of labeled nuclei for each age group is the mean number of Fos nuclei found in the cervical and lumbar spinal cord. A)This graph depicts Fos labeled nuclei located in the superficial layers. Saline treated animals showed no labeling. B) This graph describes Fos labeled nuclei found in the deeper layers. There was only a trend for the number of Fos labeled nuclei to increase with increasing age following the formalin injection.

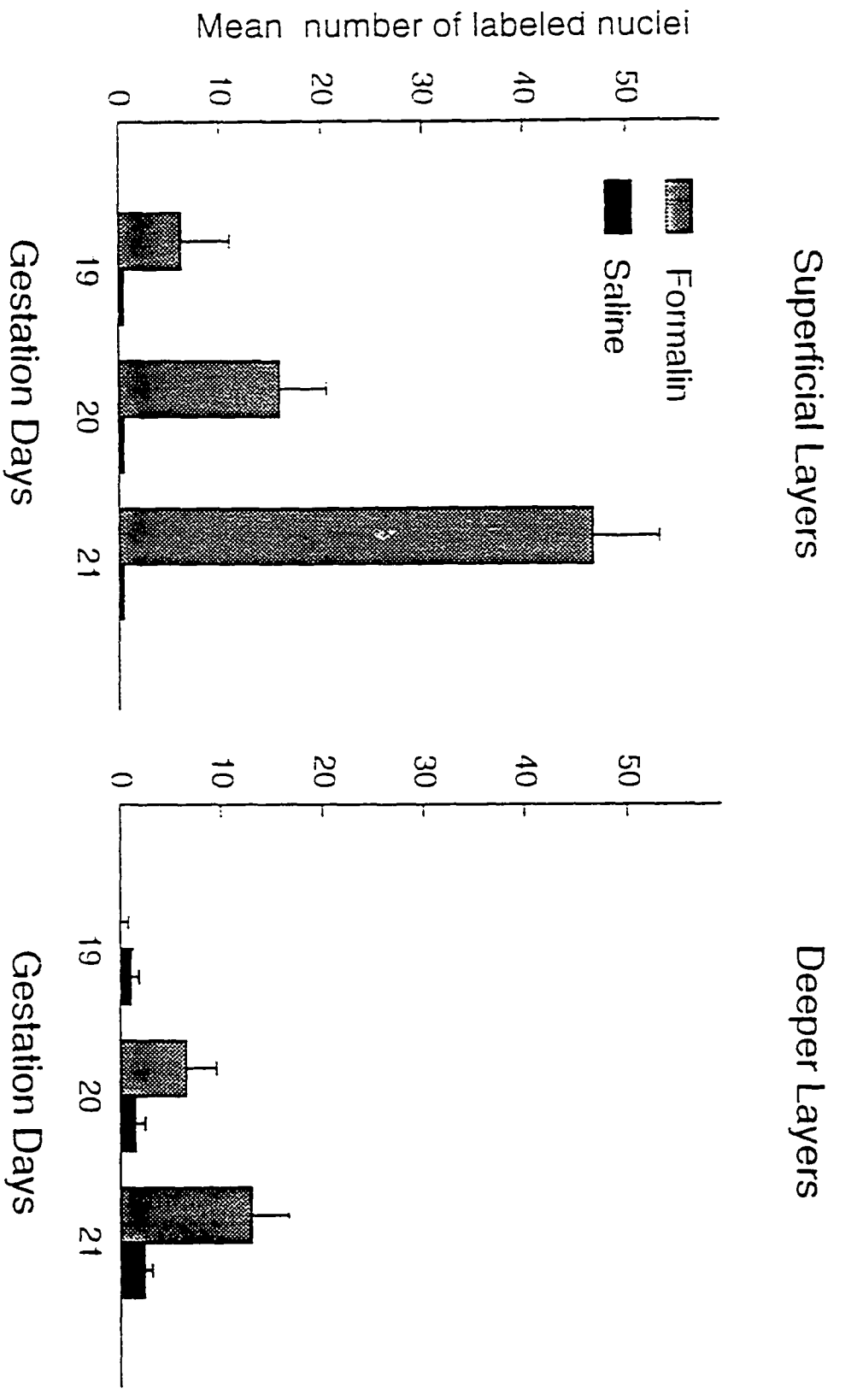


Figure 15. Histograms of Fos labeled nuclei found in GD 19, 20, and 21 following either formalin or saline injection. Raw scores, not the transformed difference scores, are used to construct these graphs. Graphs A and B describe the Fos labeling found in the stimulated side in the superficial layers (A) and deeper layers (B). The bottom 2 graphs depict Fos nuclei found in the non-injected side in the superficial layers (C) and deeper layers (D).

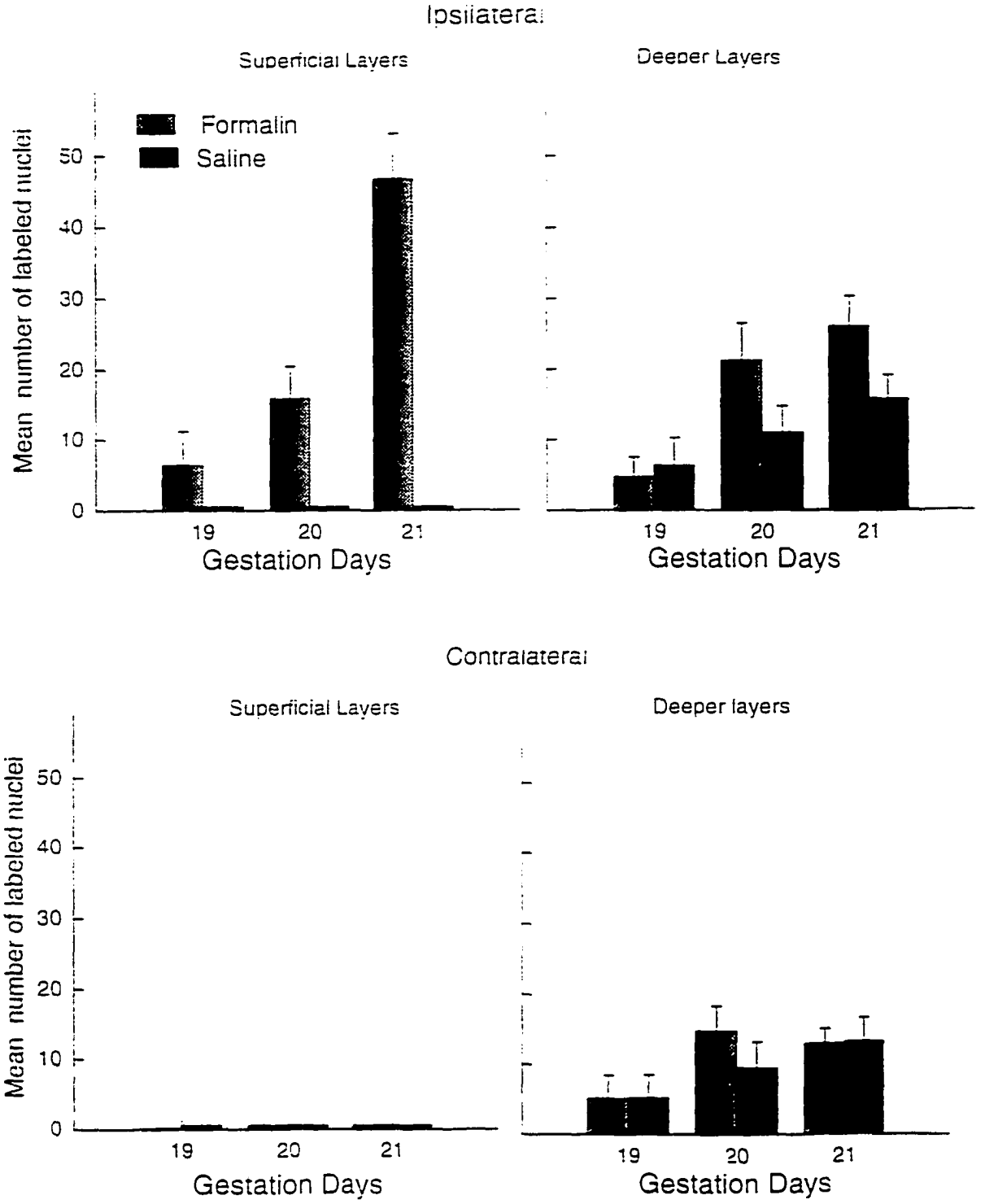
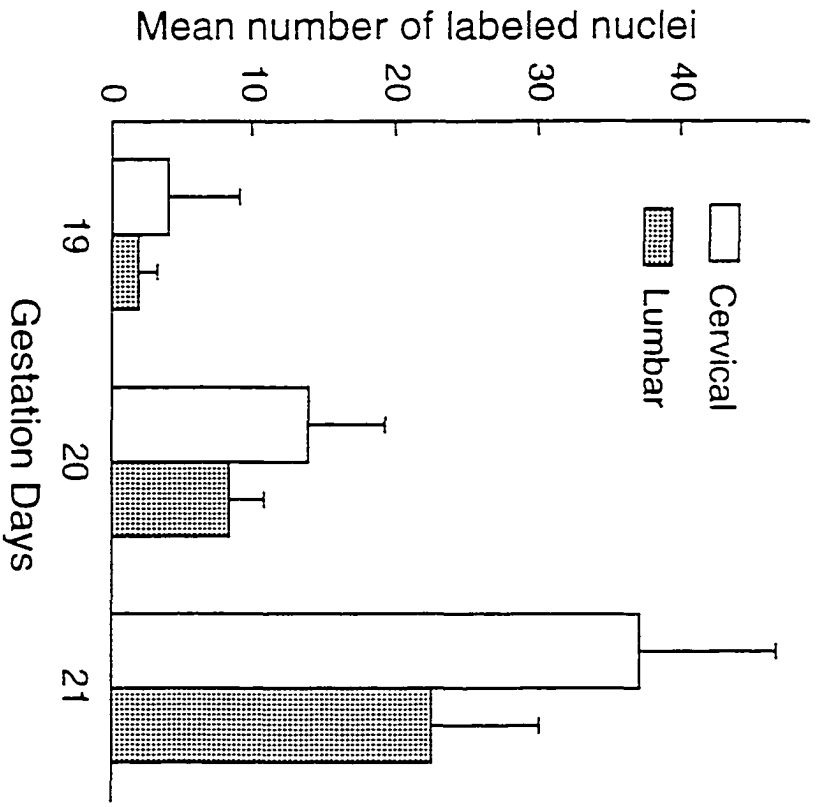


Figure 16 Histogram of Fos staining found in the cervical and lumbar cord in the 3 different gestational ages following formalin or saline treatment. For this graph, the number of labeled nuclei for each age group includes cell counts from both the superficial and deeper layers.



formalin treatment in the deeper layers of either ipsilateral and contralateral side to the injected paw in any of the fetal ages.

Cervical vs. lumbar. Overall, formalin induced greater Fos expression in the cervical cord than lumbar cord (Fig.16), (Level x Treatment). $F(1, 7) = 6.908$, $p=.034$, with no statistical difference among the 3 ages. The number of cells induced by saline injection was similar for both cervical and lumbar cord.

DISCUSSION

Behavior

Both behavioral and electrophysiological studies have shown that rat fetuses exhibit spontaneous activity starting at GD 16. Fetal rats as young as GD 17 respond to noxious stimulation and show a withdrawal reflex following a noxious input at GD 17 (Narayanan et al.,1971; Fitzgerald, 1987c). Neonatal rats respond behaviorally to formalin injection into the paw pad (Guy and Abbott, 1992; Yi and Barr, 1995), but the present study is a first report showing formalin reaction in fetal animals. We have demonstrated that the behavioral response to formalin injection develops late into gestation. The fetuses react to formalin more vigorously and specifically between GD 20 and 21. By GD 21, fetuses exhibited a wide variety of behaviors including flexing the injured limb reminiscent of the postnatal animal's paw lift. Further, fetuses at this age are able to respond specifically and differentially to the injury. For example, face wiping was seen following formalin treatment only into the forepaw, but not the hindpaw. The predominant behavior following the hindpaw injection was rear leg movement. In the adult and neonatal

rats. animals display specific responses following the formalin test (Dubuisson and Dennis, 1977; Guy and Abbott, 1992; McLaughlin et al., 1990) including paw lifting, shaking, and licking of the injured paw. However, in the fetal rat, responses to formalin such as face wiping and mouthing are not unique responses to formalin. For example, face wiping is observed prenatally following a cutaneous stimulation in the perioral area (Smotherman and Robinson, 1988) and more recently, it has been observed in fetal rats experiencing a precipitated opiate withdrawal (Jones and Barr,1996). Although different than postnatal and adult responses, fetal animals show developmentally appropriate responses to the formalin test.

Edema

The neural mechanisms of neonatal tissue inflammation may differ from those seen in the adult due to the immature immune and sensory systems (for review, Fitzgerald, 1995). Although neurogenic edema caused by activation of the C-fibers is not seen until the second week of rat's postnatal life (Fitzgerald and Gibson, 1984), noxious stimulation such as thermal and mechanical inputs and formalin induce edema from birth (Yi and Barr, 1995). In the adult the formalin effect is mediated specifically by C-fiber afferents (Dickenson and Sullivan, 1987), but in the maturing animals, formalin may serve as a non-specific chemical irritant that activates both Ad and C-fibers (Fitzgerald, 1995). In the present study, GD 19, 20, and 21 fetal animals showed swelling in the formalin injected paw with no visible difference among the 3 ages. Although GD 19 fetuses showed little behavioral response following the formalin injection, edema was observed at this

age, suggesting that mechanisms responsible for edema develop before other behavioral responses to the formalin appear.

Our behavioral findings together with the presence of edema show that fetuses as young as GD 19 do respond to a noxious peripheral input, but as the fetuses approach the completion of the gestation period, there is a marked increase in their ability to react to a noxious stimulus.

Anatomy

The anatomical findings show that at least by GD 20, fetuses are able to express C-*fos* gene in the spinal cord following the formalin injection. These findings parallel the electrophysiological and anatomical maturation of dorsal horn cells and the nociceptors. Neurogenesis of projection and interneurons in the substantia gelatinosa is completed well before birth, between GD 14 and 16 (Nornes and Das, 1974), but the axonal and dendritic development of the cells found in this area occurs later, between GD 15 - 21, with considerable postnatal maturation (Bicknell and Beal, 1984; Fitzgerald et al., 1991). On the other hand, the interneurons in substantia gelatinosa initiate their axo-dendritic growth just before birth, at GD 21 (Bicknell and Beal, 1984). Consistent with these reports, our results showed a large increase in the number of Fos labeled nuclei between GD 20 and 21. The increase was predominantly in the superficial layers with some increase in the deeper layers of the dorsal horn at GD 21. It is possible that the large increase seen between GD 20 and GD 21 is due to late maturing interneurons as well as the development of nociceptors found in the dorsal horn late into gestation. Ad and

polymodal C-fibers reach the white matter superficial to the dorsal horn on GD 19, penetrate the superficial dorsal horn on GD 19.5 - 20, and by GD 20, they are found in the inner laminae II (Fitzgerald, 1987).

Our findings further suggest that the increases in the behavioral response and the number of Fos stained nuclei observed with increasing fetal age are evidence for the rapidly maturing nociceptive circuit at the spinal level. The number of Fos cells observed is dependent on the stimulus intensity used to evoke the *C-fos* expression both in adult and neonatal rats (Bullitt, 1990; Yi and Barr, 1995a), suggesting that a more intense stimulus evokes a greater number of Fos cells compared to a less intense stimulus. In the present study, we used an equal volume of formalin for the 3 ages. If the maturational status of the nociceptive circuitry were similar for these fetuses then, because of the smaller paw size in the younger fetuses, the same formalin volume would have produced a greater degree of nociception in the younger fetuses than in the older animals. Therefore formalin treatment would have induced a greater number of Fos labeled nuclei in the younger fetus. Our findings do not support this hypothesis, but rather demonstrate increases in the number of Fos labeled nuclei with increasing fetal ages, hence supporting the differential maturity of the nociceptive pathway in the maturing fetuses.

With an exception of the constitutive level of Fos staining found bilaterally, the pattern of formalin-induce Fos appearance in the fetal animals is similar to that seen in the newborn and in adult rat (Presley et al., 1990; Williams et al., 1990; Yi and Barr, 1995a). In the GD 21 fetus, staining was found ipsilaterally in the superficial

layers and deeper layers of the dorsal horn with a conspicuous absence from the ventral horn. In the neonatal and adult rat, formalin injection produces Fos cells in the ventral horn (Presley et al., 1990; Yi and Barr, 1995). It has been suggested that Fos stained cells found in the ventral horn are polysynaptically activated and these neurons receive inputs from the dorsal horn interneurons (Presley et al., 1990). The maturation of interneurons in the superficial layers is mainly a postnatal event (Bicknell and Beal, 1984). It is possible that at GD 21 the synaptic connections between interneurons in the superficial dorsal horn cells and the ventral horn neurons have not yet been formed or matured.

Neurogenesis in the spinal cord follows the development in the rostrocaudal direction, so that the neurons in the cervical portion of the cord are born first followed by neurons in the thoracic and lumbar regions (Nornes & Das, 1974). Our findings show that cervical cord neurons do express more Fos cells than do lumbar cord neurons and that the cervical and lumbar cord difference was greater for the superficial lamina. The cervical and lumbar cord difference was not age dependent. This may suggest that cervical cord neurons are more developed compared to the lumbar cord cells or that the forepaw is more sensitive to the formalin test in the prenatal animals. This developmental difference changes with age. In newborns, however, injection of formalin into the hindpaw induced a greater number of FLI cells than in the forepaw (Yi and Barr, 1995b).

The appearance of Fos expression in the non-superficial dorsal horn area in untreated, saline treated as well as formalin treated fetuses is intriguing. The

symmetrical appearance of Fos labeled nuclei is unique to the fetal animals and not observed in any of the postnatal, including adult, animals. The constitutive level of staining was observed in lamina III where large and small-diameter myelinated cutaneous afferents terminate (Light and Perl, 1979). These fibers are found in the dorsal horn between GD 16.5-17.5 (Smith, 1983). Because the labeling is observed in untreated animals, it is more likely that these constitutive expression of Fos in the spinal cord has some function during development in the spinal cord rather than playing a role in transmitting nociceptive signals. There is ample evidence that proto-oncogenes such as C-*fos* and C-*myb* are expressed in developing neural tissues (for reviews, Adamson, 1987; Sudol, 1988), although the function of this gene expression in embryos is still not clear. There are no studies that have examined the function of the expression of *C-fos* proto-oncogene in fetal animals of the spinal cord. *In vitro* studies using different cell lines have suggested that the appearance of Fos protein may be important in fetal animals by acting as the second messenger and by participating in the differentiation process (Greenburg et al., 1985; Curran and Morgan, 1985). Although it is reasonable to assume that the observed baseline Fos expression in the spinal cord plays a role in differentiation, more definitive and detailed explanation for this phenomenon warrants further investigation.

We have demonstrated in this study that fetuses are capable of responding both behaviorally and anatomically to painful peripheral inputs well before birth. Behaviorally, by GD 21, fetuses exhibit a variety of behaviors such as face wiping and "paw favoring" following the formalin injection. Anatomically, formalin

induces Fos stained cells in GD 20 fetuses and the number of formalin-induced Fos cells increase with increasing fetal age.

CHAPTER 4

The Suppression of Formalin-Induced- Fos Expression by Different Anesthetic Agents in the Infant Rat.

In the adult, anesthetics act by altering neuronal activity in selected regions of the central nervous system, both in the spinal cord and at supraspinal sites (Collins, 1993b; Koblin, 1990). For example, inhalational anesthetics, such as nitrous oxide and halothane, and barbiturates, such as sodium pentobarbital, act in part by altering cerebral cortex and hippocampus activity (Angel and Gartton, 1982; Gage and Robertson, 1985). In addition, inhalational agents also act on neurons within specific laminae of the spinal cord (de Jong, Corbin and Nace, 1968; de Jong, Robles and Heavner, 1970; de Jong, Robles and Morikawa, 1969; de Jong and Wagman, 1968; Namiki, Collins, Kitahata, Kikuchi, Homma and Thalhammer, 1980) but not on sensory receptors (de Jong and Nace, 1967; Takenoshita and Takahashi, 1987).

A detailed understanding of the mechanisms by which analgesics and anesthetics work is important in alleviating pain in infant patients. However, we know little about how anesthetics and analgesics work in the immature animal or human infants. Furthermore, the site of anesthetic action in the neonate has not been well described. An electrophysiological study using *in vitro* neonatal spinal cord preparations have shown that anesthetics such as isoflurane produce an antinociceptive activity at the spinal cord level (Savola, Woodley, Maze and Kendig, 1991). However, there are no studies that have examined anesthetic effects in immature animals *in vivo* .

The proto-oncogene c-*fos* is a member of a family of immediate early genes that is rapidly induced within the central nervous system following neuronal depolarization (Morgan and Curran, 1989; Sheng and Greenberg, 1990). Because the Fos oncoprotein can be visualized in single neurons, it has become an important

method in examining neural pathways that are activated by a variety of stimuli such as seizure (Morgan, Cohen, Hempstead and Curran, 1987), dehydration (Sagar, Sharp and Curran, 1988), light (Rea, 1989), hypothermia (Joyce and Barr, 1990) and pain (Abbadie and Bessson, 1993; Bullitt, 1989; Bullitt, 1991; Hunt, Pini and Evan, 1987; Menetrey, Gannon, Levine and Basbaum, 1989; Presley, Menetrey, Levine and Basbaum, 1990; Williams, Evan and Hunt, 1990a; Yi and Barr, 1995). In the study of nociceptive pathways, the Fos protein has been especially useful because the Fos expression is evoked in specific areas of the spinal cord that are known to be involved in transmitting nociceptive information both in the neonate (Williams, Evan and Hunt, 1990b; Yi and Barr, 1995) and adult rat (Presley et al., 1990).

The goal of this experiment was to determine whether some of the commonly used anesthetic agents suppress c-*fos* expression following the formalin test in the 3-day-old rat. The anesthetic agents were methoxyflurane, acepromazine, hypothermia, and a ketamine-xylazine mixture. These four treatments were chosen because they are commonly used in laboratories and veterinary medicine to induce anesthesia both in the adult and infant animals. Acepromazine is a sedative, but it is widely used as a preanesthetic drug.

Methods

The pups were removed from the dam and were randomly assigned to one of the following anesthetic conditions: methoxyflurane (n=4), a mixture of ketamine and xylazine (n=5), acepromazine (n=5) or hypothermia (n=7). Pups within a single treatment condition came from different litters. Methoxyflurane was administered

by inhalation; the ketamine-xylazine combination (35 mg/kg of ketamine hydrochloride and 3.0 mg/kg of xylazine: 5 ml/kg) and acepromazine (50 mg/kg) were injected intraperitoneally (1 ml/100g). Doses for ketamine-xylazine mixture and acepromazine were chosen from pilot experiments. These doses are the lowest possible amounts of drug at which animals are anesthetized for 2 hours without receiving a second injection. Hypothermia was induced by immersing the entire body caudal to the neck in an ice cold water with crushed ice. When the anesthetized pups were unresponsive to handling, 10 µl of formalin (10%) (McLaughlin, Fanselow and Cramer, 1990; Yi and Barr, 1995) was injected into the plantar pad of the right hindpaw. The control animals included both a saline injected group (n=3) that was treated identically to the anesthetized groups except that saline was injected into the hindpaw, and awake animals receiving formalin injection to serve as non-anesthetized controls (n=3). Another study that examined the Fos expression in awake 3 day old pups was conducted in our laboratory at the time of this experiment, and because some of the tissue from these two experiments were processed together, and for ethical reasons, we used this group as our non-anesthetized control for this experiment (Yi and Barr, 1995). Pups were kept anesthetized for 2 hours following formalin injection. Methoxyflurane exposed pups needed supplemental exposures to the drug to remain anesthetized for the 2-hour period. However, both acepromazine and ketamine-xylazine treated pups were anesthetized for two hours at the above doses. Animals treated with hypothermia were placed on an ice pad until they were sacrificed. All pups with an exception of cold treated animals were kept in an incubator to help maintain the normal body

temperature until the perfusion time. All animals were perfused at 2 hours post-injection.

Four immunocytochemical assays were performed in this experiment. For each set of immunocytochemical reactions, tissue from at least two treatment groups and both control groups (saline and unanesthetized formalin injected groups) was processed together. Except for these constraints the tissue was assayed in a non-systematic manner to avoid biasing the data due to possible day to day assay variability. In prior work we found no sign of variability in the number of Fos labeled cells from assay to assay (Yi and Barr, 1995).

Results

Behaviorally, the ketamine and xylazine combination and the cold treated animals showed more profound signs of anesthesia than did either methoxyflurane or acepromazine administered pups. Behavioral anesthesia is defined as a complete loss of righting reflex, total unresponsiveness when handled and complete absence of spontaneous motor activities. These former groups of pups were completely unresponsive to handling, and were purplish-blue in color. Methoxyflurane and acepromazine administered animals were also unresponsive to handling, and, although their respiration rate was not counted, they appeared to be breathing slightly more rapidly than the other two treatment groups, and both were pink in color. Edema of a varying degree occurred in the formalin injected paw of all treatment groups, but the behavioral responses typically seen in the formalin test such as paw lifting and licking (Abbott, Melzack and Leber, 1982; Dubuisson and

Dennis, 1977; McLaughlin, Lichtman, Fanselow and Cramer, 1990) were not observed in any of the anesthetized pups. The edema was more severe in the acepromazine and methoxyflurane treated animals than the ketamine-xylazine or cold treated groups, which showed little swelling. The control animals that received saline injections did not exhibit edema in the injected paw nor any other behavioral responses associated with the formalin test. Awake animals injected with formalin showed paw lifting and edema in the injured paw at this age as shown previously (McLaughlin et al., 1990; Yi and Barr, 1995).

The Fos staining was exclusively ipsilateral following formalin injection. Although the entire lumbar portion of the cord was sectioned, the highest number of Fos stained cells were observed at the enlargement, L 3- L 4. The saline injected animals did not demonstrate any staining. Depending on the treatment conditions, the number of animals exhibiting Fos labeled nuclei varied. Of 4 pups treated with methoxyflurane only one exhibited Fos labeling. On the other hand, unanesthetized, acepromazine and ketamine-xylazine demonstrated a more consistent Fos labeling, occurring in all the animals.

The one-way analysis of variance (ANOVA) performed on the total number of stained nuclei per treatment condition yielded a statistically significant results. $F(4,19)=9.34$, $p<.05$. Fisher's LSD post hoc tests revealed that ketamine-xylazine treated animals and the unanesthetized control group each showed significantly more staining than did methoxyflurane, cold or acepromazine treated animals. The ketamine-xylazine and the non-anesthetized groups did not differ from each other (Figure 17 and 18). The pattern of fos staining was not strikingly different across

Figure 17. The mean number (±SEM) of formalin-induced Fos-stained nuclei following different anesthetics found in different regions of the cord. Total represents the sum of the labeled nuclei for each anesthetic. Data from all animals are included. For example, in methoxyflurane-treated group, the Fos staining was only apparent in one animal, but the mean presented in this graph is based on 4 pups.

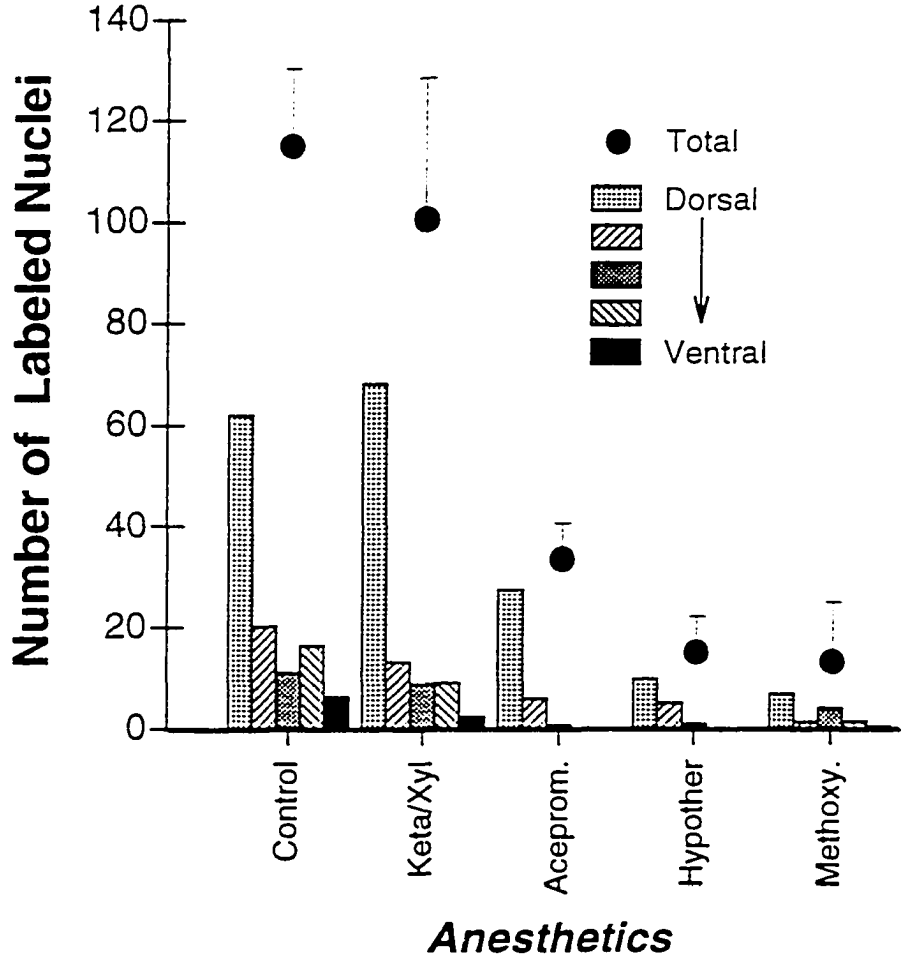
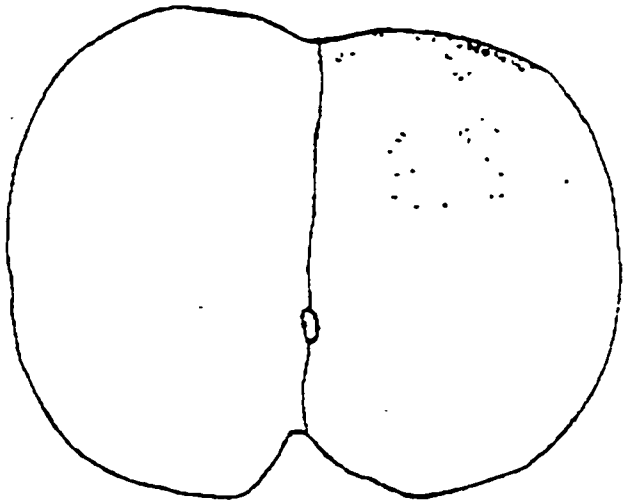
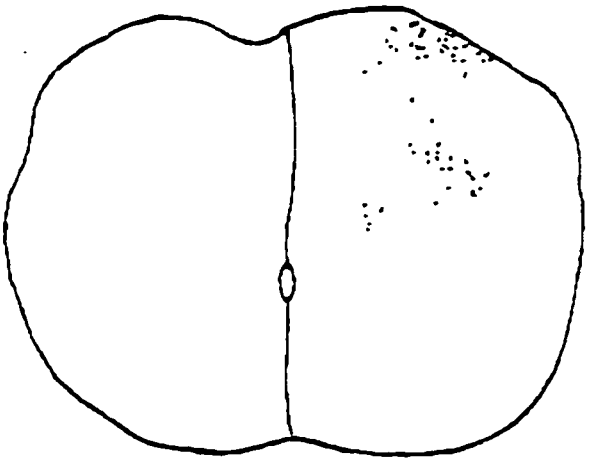


Figure 18. Camera lucida drawings of formalin-induced Fos-stained nuclei in the lumbar spinal cord. The number of Fos-stained nuclei in ketamin-xylazine-treated pups is not different from the unanesthetized control group. Note that the methoxyflurane group is not represented because sections from only 1 pup showed staining.

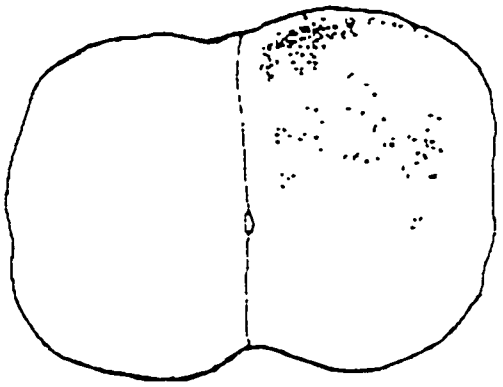
Acepromazine



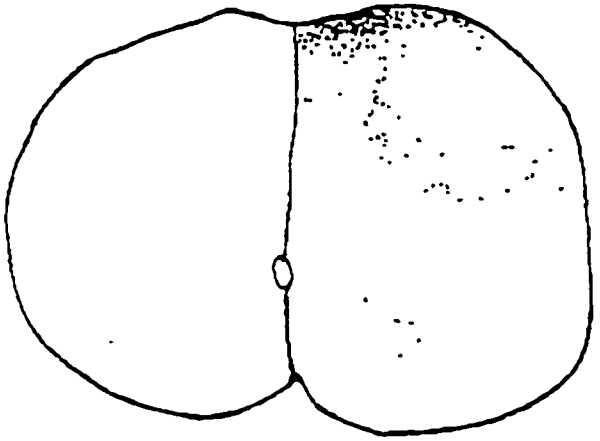
Cold



Control



Ketamine-xylazine



anesthetic conditions with an exception that fewer stained cells are found in hypothermia and acepromazine conditions than ketamin-xylazine or unanesthetized control.

When observed, the Fos staining pattern was similar to that obtained following morphine treatment in adult rats (Presley et al., 1990): the medial portion of the superficial laminae had the most number of stained cells and nuclei in the deeper layers of the dorsal horn failed to express Fos staining (Figure 18).

The variability in the staining among methoxyflurane and cold exposed animals cannot be attributed to the variability in the immunocytochemical reactions. When either methoxyflurane or cold treated groups was assayed together with ketamine-xylazine administered groups, staining was seen in the ketamine-xylazine exposed group, but not in the methoxyflurane or cold treated groups.

Discussion

Despite the wide use of activation of c-*fos* gene as an anatomical marker, the exact role of c-*fos* gene in different neural systems is not known. However, in the pain circuitry both in adult and immature rats, Fos labeled cells are found in the spinal cord regions that have been associated with transmission of pain signals (Hunt et al., 1987; Bullitt, 1989; Presley et al., 1990; Yi and Barr, 1995) . For example, Fos stained cells are found in laminae I, II and V which is consistent with electrophysiological and anatomical studies (for review, Besson and Chaouch, 1987). In addition, some of the cells in the ventral horn spinal cord have also been

found to be labeled with the Fos protein following formalin in the adult rat (Presley et al., 1990). Our results show that, in the infant rat as in the adult, anesthetic agents suppress the appearance of Fos protein in the spinal cord dorsal horn cells. The depression of spinal activity was apparent in methoxyflurane and cold treated animals where no Fos stained nuclei was observed in most of the animals. In adults, it has been shown that volatile and gaseous agents markedly depressed excitatory postsynaptic potentials recorded in the ventral root (de Jong et al., 1968), do not affect the activity of primary afferents but that they act on neurons within specific laminae of the lumbar cord (Heavner, 1975). Our data cannot determine whether in infant rats the suppression of c-*fos* expression is produced by effects on the primary afferents or at the level of the spinal cord. In addition to a direct action at the spinal level, the activation of the descending inhibition from the brain has also been suggested as a possible mechanism by which inhaled agents depress activity in the spinal cord (de Jong and Nace, 1967). Therefore, the elimination of Fos oncoprotein expression could be due by activation of the descending inhibitory paths. However, it seems unlikely that methoxyflurane exerts its effect through descending inhibition because it is not likely that this inhibition occurs at this age (Fitzgerald and Koltzenburg, 1986; Barr, Miya and Paredes, 1992).

Hypothermia may also suppress the formalin-induced-Fos by acting at the spinal cord level as in the mature animal. Research has indicated that hypothermia blocks sensory conduction by cooling nerve fibers (Gasser, 1931). The cooling of nerve fibers causes a general decrease in nerve excitability such that amplitude and conduction velocity of an action potential are markedly reduced if not obliterated (Kida, Takano, Kitagawa and Tsuji, 1994). As expected, our data demonstrate that

hypothermic treatment depressed the c-*fos* expression in the infant rat. The complete suppression of formalin-induced Fos immunoreactivity in the 57% of cold treated pups suggests that in these animals, the general decrease in nerve excitability prevented nociceptive and possibly other somatosensory information from reaching the spinal cord.

In some animals, hypothermia and to a lesser extent methoxyflurane, failed to eliminate totally the Fos staining. We hypothesize that this may be due to differences in the level of anesthesia among the pups. It is difficult if not impossible to equate the level of anesthesia between animals. Although a careful observation of the pups produced no discernible differences in the level of anesthesia among them, behavioral observations alone may not be sufficient in assessing the depth of anesthesia.

Because acepromazine maleate is not an anesthetic but a sedative (Watney and Pablo, 1992) that is commonly used as preanesthetic medication, it was expected that some Fos staining would be expressed in response to the formalin test as our data showed. When compared to the unanesthetized group the acepromazine administered pups exhibited approximately 30% of the number of Fos stained nuclei of the control. The partial suppression of Fos expression by acepromazine suggests that it may induce anesthesia-like state by depressing some of the spinal cord neurons, but it may also act at supraspinal sites, and on non-pain systems.

Electrophysiological and *in vivo* studies have shown that xylazine, a non-narcotic sedative, is an alpha-2-adrenoceptor agonist that produces antinociceptive activity in the spinal cord (Kendig et al., 1991; Kyles et al., 1993; Savola et al., 1991). Ketamine, a NMDA receptor antagonist, (Brockmeyer and Kendig, 1995;

Lodge and Anis, 1984; Woodley and Kendig, 1991) blocks activity of the dorsal horn cells (Conseiller, Benoist and Hamann, 1972; Kitahata, Taub and Kosaka, 1973) as well as neurons in the thalamus and the medullary reticular activating system (Collins, 1993a). The ketamine-xylazine results were therefore unexpected when the ketamine and xylazine combination was completely ineffective in eliminating Fos nuclei at the level of the spinal cord. This finding was even more surprising because ketamine-xylazine treated pups seemed to be deeply anesthetized. It can be only postulated at this time that the pharmacodynamic mechanism of ketamine and xylazine may differ in the maturing rat pup, so that ketamine-xylazine may exert its analgesic and sedative effects mainly in the brain sites and not in the spinal cord at this age. This somewhat surprising result further illustrates the importance of *in vivo* studies to describe any differences between the adult and immature animal.

One issue that needs to be addressed in this study is the differentiation between analgesic and anesthetic state of the animals. Analgesia refers to relief of pain without loss of consciousness, but anesthesia refers to loss of all sensation. When animals are under general anesthesia, it is unlikely that the somatosensory system including pain perception remains activated and only motor system is affected by the anesthetics. In the present study, although unlikely, it is still possible that pain pathways are activated but the animals are incapable of responding to the formalin test. This offers an alternative explanation for the appearance of Fos labeled cells especially in animals exposed to ketamine-xylazine mixture.

It has been previously noted that inflammation (Kress, Koltzenburg, Reeh and Handwerker, 1992) or swelling of the skin (Handwerker, Kilo and Reeh, 1991)

itself activates primary afferents in the dorsal horn. Our present study clearly illustrates that the Fos protein is expressed in response to activation of the primary afferents rather than edema of the paw. Animals given the ketamine-xylazine mixture showed little if any swelling of the injured paw, yet the number of Fos stained cells was not any different the unanesthetized control group.

Our laboratory has shown that in the 3 day-old pups increasing formalin volume produced more staining in the deeper layers of the lumbar spinal cord (Yi and Barr, 1995). In acepromazine and cold treated pups that demonstrated Fos staining, much of the staining in the deeper layers of the dorsal horn was eliminated. This evidence suggests that even in young animals the dorsal horn cells are differentially responsive to different manipulations of nociceptive system, a finding that is consistent with the adult literature (Presley et al., 1990).

In summary, in the infant rat, hypothermia and methoxyflurane were most effective in suppressing the formalin-induced Fos expression in the dorsal horn of the spinal cord. The combination of ketamine and xylazine failed to eliminate expression of the Fos oncoprotein in the spinal cord suggesting that, unlike cold or methoxyflurane treatment, ketamine-xylazine may act on sites located rostral to the spinal cord. The results also demonstrate the utility of the c-*fos* method for demonstrating anatomically sites involved in modulation of noxious stimulation.

CHAPTER 5:

Effects of Intracerebroventricular Morphine Administration on Behavior and Fos-like-Immunoreactivity in the Spinal Cord of Infant Rats

INTRODUCTION

Morphine administered directly into the brain exerts its analgesic effects on spinal nociceptive neurons at least in part through the descending inhibitory control system (Basbaum and Fields, 1984). Anatomically, descending axons in the spinal cord are present at birth (Leong et al., 1984), and by postnatal day 6, their distribution is comparable in number to the adult rat (Fitzgerald and Gibson, 1987). Despite its early anatomical presence, when measured electrophysiologically, this system is functionally immature during the first 2 weeks of rat's postnatal life (Fitzgerald and Gibson 1987). Behavioral studies have also confirmed the relative lack of descending inhibitory influence at more caudal, but not rostral spinal levels in the young animal (Giordano and Barr, 1987; Barr et al., 1992; Tive and Barr, 1992); opiate-induced analgesia was not observed either in the hindpaw or tail, but was apparent in the forepaw of 3 day old rat pups. Bulbospinal descending pathway uses different neurotransmitters such as serotonin and norepinephrine (Bregman, 1987; Kurashi et al., 1983). The development of these two transmitter systems follow different time courses and the distribution found within the spinal cord differs; serotonergic fibers are found at an earlier age in more rostral spinal cord levels compared to caudal levels, but noradrenergic fibers are found uniformly in the spinal cord by PD 10 (Bregman, 1987). The functional immaturity of the descending inhibitory control has been attributed to the slow appearance of serotonergic system. Because activation of the descending influence is one of the major mechanisms by which opiates produce their analgesic effects at the spinal level, understanding the developmental profile of the descending inhibition system has important clinical implications. However,

there are no studies that have examined the extent to which the system is functionally immature at different levels of the cord. A first goal of these studies was to determine the functional maturity of this system at different levels of the cord. This was studied by examining whether an opiate administered to the brain suppresses activities of the nocireponsive spinal neurons at different spinal levels, as measured by Fos immunoreactivity.

It has been reported in the mature animal that bulbospinal system exerts powerful analgesic effects on both supraspinally and spinally mediated nociceptive behavior. Limb withdrawal or hot plate tests are supraspinally mediated behaviors. In contrast, tail flick or limb withdrawal against intense heat (> 52° C) seems to be spinally mediated (Jensen and Yaksh, 1986). Therefore, in the mature animal, bulbospinal system plays a role in pain modulation, and also plays a direct role in modulating motor outflow at the spinal level (Jensen and Yaksh, 1986). However, in the developing animal, it has been consistently found that morphine is not analgesic against intense heat applied to the hindpaw (Giordano and Barr, 1987). Therefore, a second goal of the present studies was to determine if there is a difference in developmental profile of analgesia of supraspinally mediated behavior (reaction to formalin injection) and spinally mediated behavior (limb withdrawal against intense heat).

Fos expression has been extensively used as a functional marker of neuronal activity in pain circuitry. Despite some limitations, such as poorly understood functional significance of the upregulation of c-*fos* gene expression following nociception, the use of the c-*fos* gene expression as a marker provides several advantages. A population of neurons can be easily observed at a single cell

resolution: cells expressing Fos can be counted therefore quantification of the results are possible: and behavior following nociception can be observed. Present studies employed Fos immunoreactivity as a functional and anatomical marker for neuronal activity in response to nociception.

METHODS

Drug administration

Morphine sulfate (H. Schein) was diluted in distilled water and injected intracerebroventrically (ICV). The procedure for i.c.v injection was modified from Ellis et al. (1983). Drug was loaded into a 5-µl syringe with a 30-gauge needle that was fitted with a guard. The length that the needle extended beyond the guard corresponded to the depth of the cerebellomedullary cistern (cisterna magna) at each age. The pup was hand-held and gently restrained. The skull was punctured and 2 µl of drug was injected over a 1 minute period.

Formalin stimulation

Thirteen litters of 3 and 14 day old rat pups were given either vehicle, 3 µg, or 10 µg morphine intracerebroventrically. Fifteen minutes later, 10 (3 day old pups) or 20 µl (14 day old rats) of 10% formalin was injected in the plantar surface of the right forepaw or the hindpaw. Immediately following the formalin injection, animals were observed every minute for a total of 30 minutes. Each pup's response to the formalin injection was scored following the method of Dubuisson and Dennis (1977). The ratings ranged from 0 to 3, the higher number indicating more severe reaction to formalin. A score of 0 was assigned if the injected paw was comfortably

placed on the floor, without favoring or guarding. A rating of 1 indicates that the injured paw was in contact with the floor with slight favoring of the injected paw. A score of 2 was assigned if the animal lifted the injected paw ("paw lift") and 3 indicates that the injected paw was lifted and licked ("paw lick"). For each animal, these ratings were summed. A maximum total behavioral score possible was 90. Both the paw stimulated and the drug doses were within-subjects variables and the tissue from all the conditions was processed together.

Thermal stimulation

Three-and 14 day-old pups were administered morphine intracerebroventrically (0, 0.3, 1.0, 3.0, and 10 μg) 15 minutes prior to the peripheral stimulation. Either the forepaw or the hindpaw was immersed in a hot water bath (52 ° C) and withdrawal latencies were recorded. If the withdrawal latencies were less than 5 seconds, the paw was held in the water bath for a total of 5 seconds. Thus, irrespective of conditions, all pups were exposed to the hot water bath for a total of 5 seconds at each testing period. The cut-off latency was 5 seconds. Animals were tested in this manner at 5 minute intervals for 15 minutes. This protocol was followed to insure the induction of c-*fos* expression because the intensity or duration of stimulus has shown to be related to the number of Fos labeled nuclei observed in adult and infant animals (Bullitt et al., 1990; Yi and Barr, 1995a). Immersion of either paw in a neutral temperature (32 °C) water bath did not elicit the withdrawal behavior.

Morphine doses and paw stimulated were within-subjects variables and age was the between-subject variable. Animals were sacrificed and immunocytochemistry

was performed as described in the general methods section. Tissue from all conditions (5 morphine doses, cervical and lumbar segment and the 2 age groups) was processed together.

RESULTS

Formalin

Behavior Both 3 and 14 day old pups treated with vehicle responded to formalin injection, often favoring the injected paw and paw lifting and licking as previously reported in the neonate (Guy & Abbott, 1992) and adult (Coderre et al., 1993).

Morphine treated animals were generally sedated, especially at the 10 μg of morphine dose at both ages. As can been seen from Figure 19, forepaw stimulation in the 3 day old pup produced low "pain" responses. Therefore, even though morphine reduced pain scores, this effect was not statistically significant. In the hindpaw of 3 day old rat, morphine reduced pain scores only at the high dose ($p<0.05$). At 14 days of age, morphine administered intracerebroventrically significantly reduced pain scores in a dose-dependent manner both in the forepaw and hindpaw, $F(2, 22)=9.16$, $p<0.05$. Although the analysis suggests that morphine is less effective in reducing pain scores in the forepaw of the younger animal, that interpretation is clouded by the different baselines between the two age groups; the amount of pain behavior exhibited following the morphine treatment was similar for the two ages for the forepaw.

Following morphine treatment, pain scores in the hindpaw were consistently greater than for the forepaw at both ages ($p<0.05$), indicating that the analgesic effects were greater for the forepaw. At 10 μg, morphine nearly completely

eliminated pain related behaviors in the forepaw. In contrast, morphine failed to reduce pain scores to the same extent in the hindpaw at either age. *C-fos Expression* Morphine suppressed Fos immunoreactivity in a dose-dependent manner for the two ages. There was no main effect of age or significant interaction of age with morphine (p's > .05); thus, the pattern of Fos reduction by morphine was not different for 3 and 14 day old animals (Fig.20). Morphine decreased the number of Fos labeled nuclei in the cervical cord to a greater extent than in the lumbar cord, $F(2, 16) = 5.25$, $p < 0.05$, suggesting that, overall, Fos labeled nuclei in the cervical cord were more sensitive to the effects of morphine. Analysis of the percent reduction from the saline control in different areas of the cord showed that the reduction in the superficial layers (areas A and B) was similar to that observed in deeper dorsal horn (areas C and D) and ventral horn (area E) cells, unlike in the adult animal where there is greater reduction in the deeper dorsal horn and ventral horn of the spinal cord compared to the superficial layers.

Thermal stimulation

Behavior There were no differences in the baseline withdrawal latencies between 3 and 14 day old pups. For the forepaw, morphine significantly increased the withdrawal latencies in a dose-dependent manner (Fig. 21) for both the 3 and 14 day old animal, $F(4, 24) = 3.93$, $p<0.05$. Morphine increased the withdrawal latencies to a greater extent in the 3 day old pups than in 14 day old animals. This effect was particularly true at higher doses (3 and 10μg). At these two doses, 3 day old pups failed to withdraw their paws from the water bath. Morphine did not produce any analgesia against the thermal stimulus in the hindpaw at either age.

Figure. 19. These graphs summarize the effects of morphine on cumulative pain scores following formalin injection into either the forepaw or hindpaw. Note the low baseline scores in the forepaw of the 3 day old pups. Morphine was effective in inducing analgesia in both paws, at both ages. Unlike in the hindpaw, morphine nearly completely inhibited pain behaviors at higher doses of morphine in the forepaw at both ages.

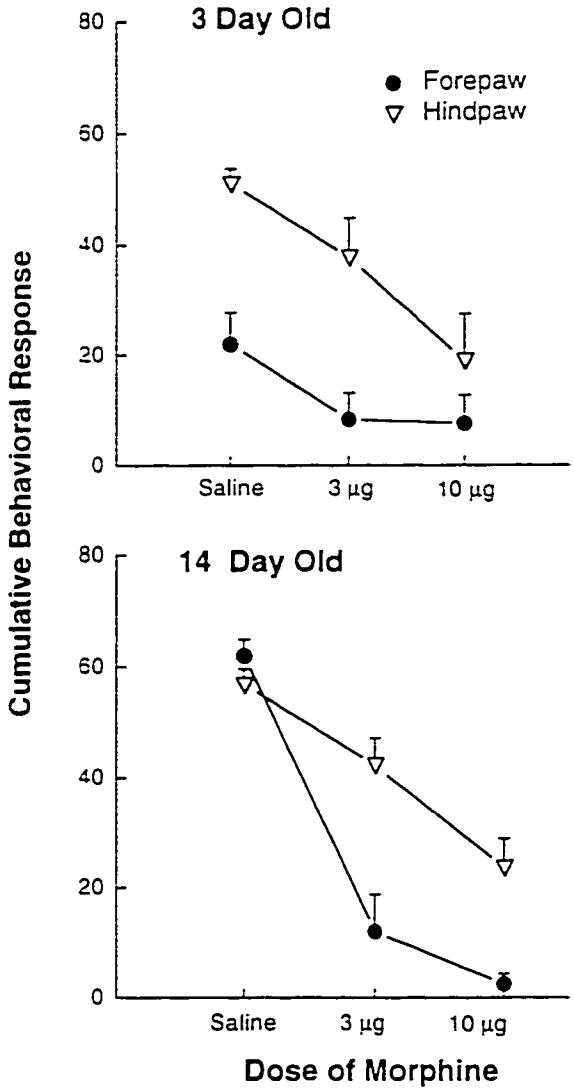


Figure 20. The mean number (±SEM) of Fos immunoreactive nuclei observed following different doses of morphine. Fos was induced by 10 µl (20 µl for 14-day-old pups) of 10 % formalin. Morphine reduced the number of Fos labeled nuclei in a dose related manner, in most conditions. Morphine had a greater effect on Fos labeled nuclei observed in the cervical cord, compared with those observed in the lumbar spinal cord.

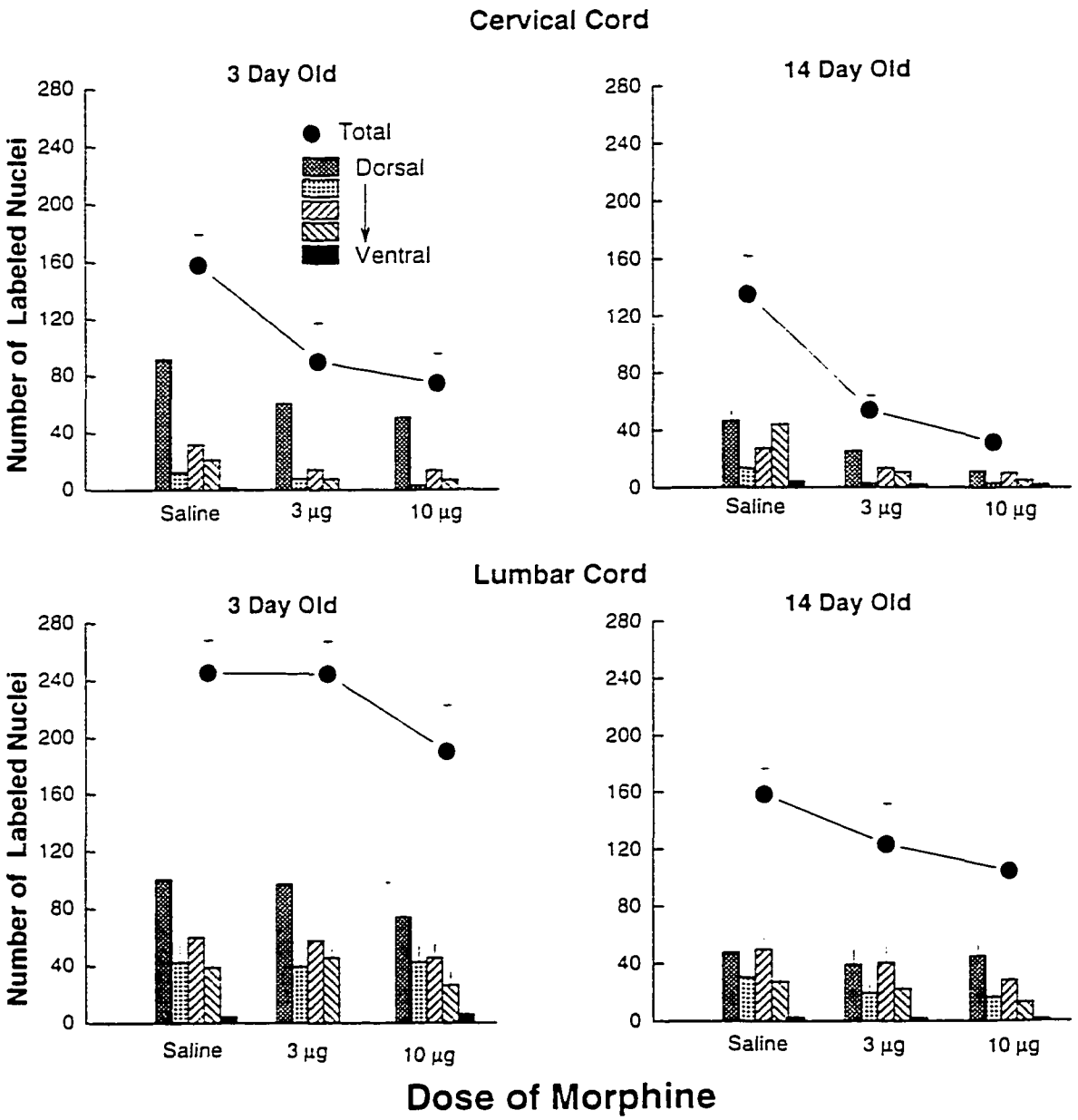


Figure 21. These graphs summarize the effects of morphine on the withdrawal latencies following immersion of either the forepaw or hindpaw into a hot water bath. Morphine induced analgesia in the forepaw, but morphine did not produce analgesia in the hindpaw at either age.

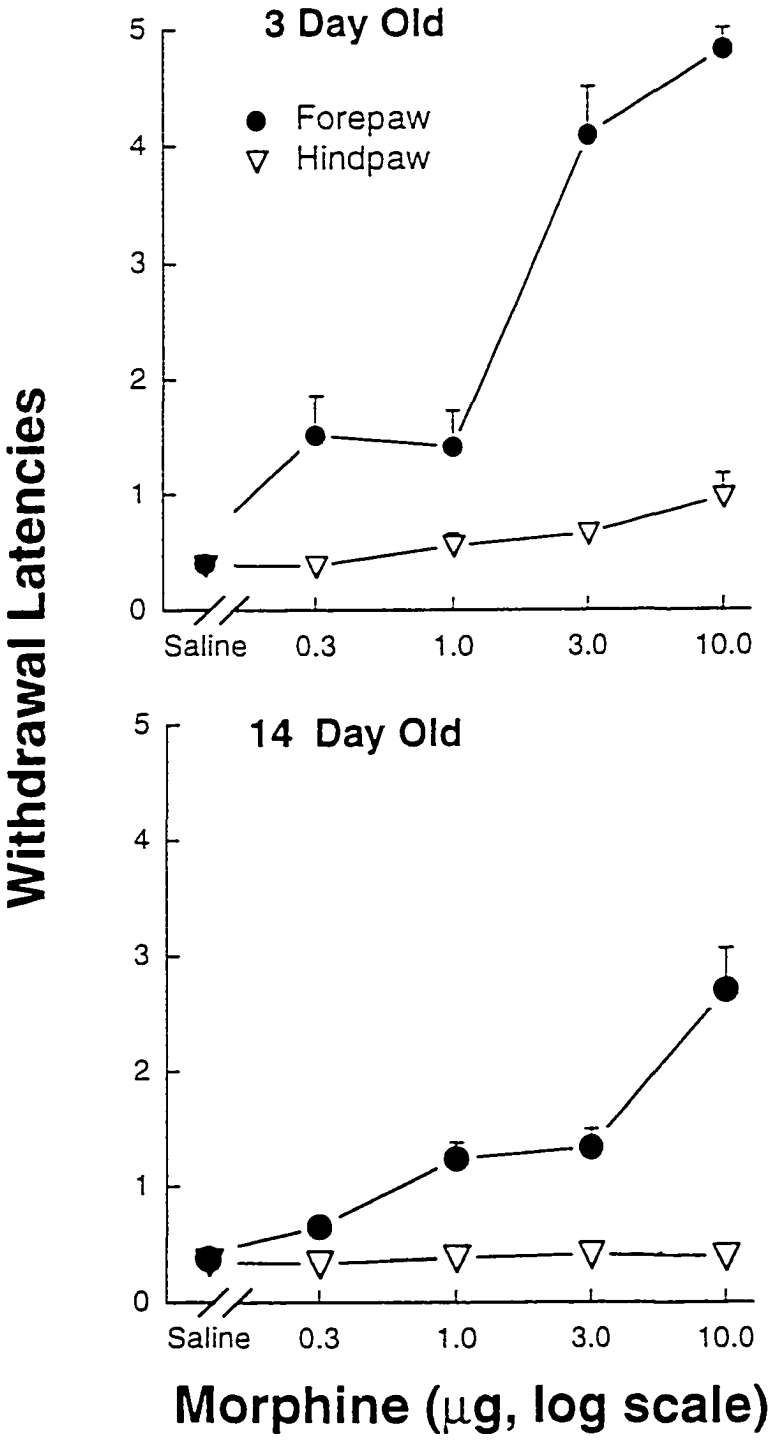
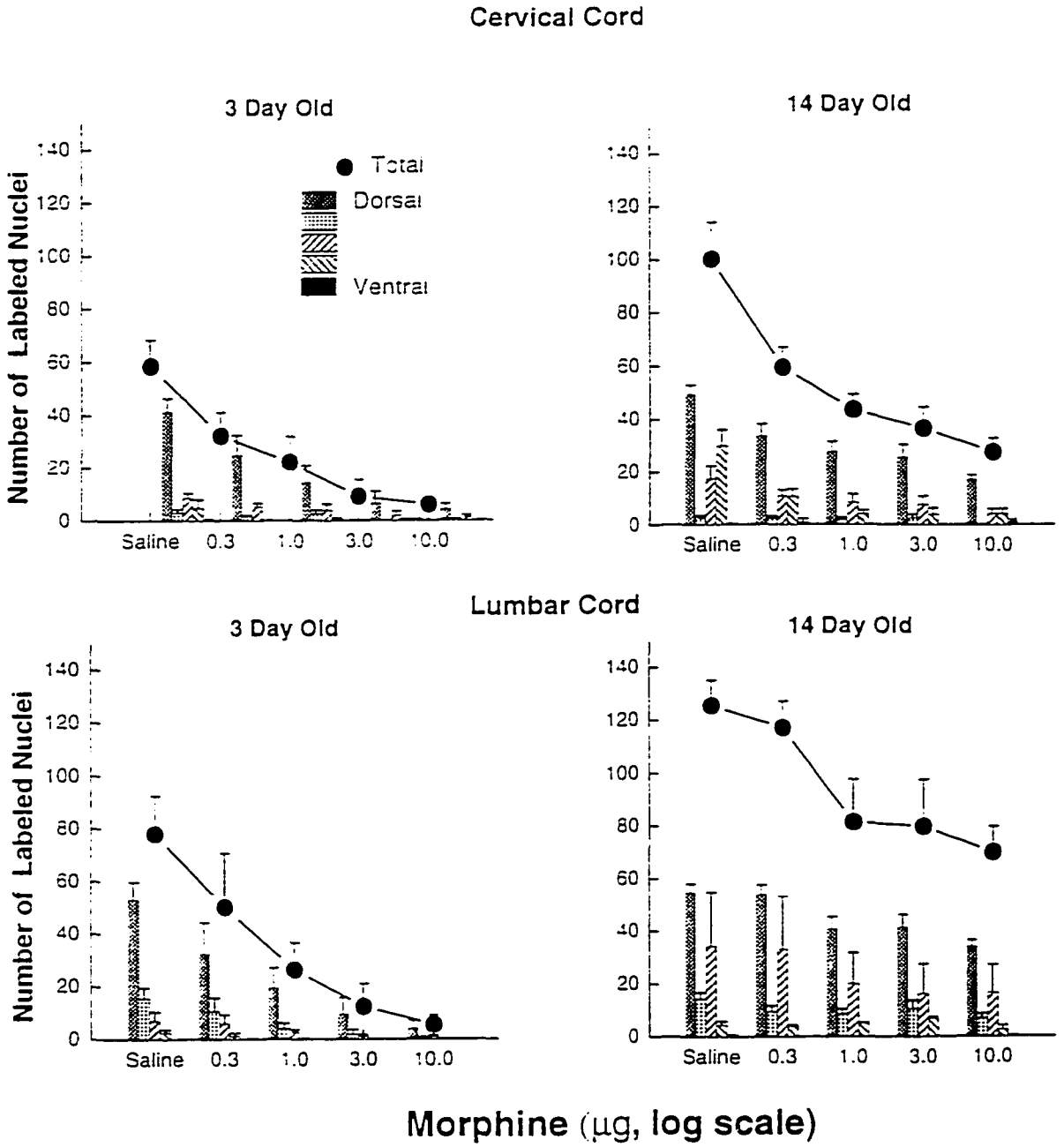


Figure 22. The mean number (±SEM) of Fos immunoreactive nuclei observed following different doses of morphine. Fos was induced by immersing either paw into a hot water bath. Morphine reduced the number of Fos labeled nuclei in a dose related manner. Note the similarity in the shape of the curve between cervical and lumbar spinal cord at 3 days of age.



C-fos Expression Morphine reduced the number of Fos labeled nuclei to a similar extent at the two levels of the cord in a dose dependent manner (Fig. 22). However, the suppression pattern between the two ages was different, and morphine was more effective at 3 days of age. In the younger animals, morphine reduced the number of Fos labeled nuclei in a dose dependent manner, and at 10 μg of morphine, there was nearly complete suppression of Fos immunoreactivity. At 14 days of age, Fos was inhibited in a dose dependent manner, but even at the highest dose of morphine, Fos immunoreactivity was present.

In the 3 day old pup, reduction of the number of Fos labeled nuclei was greatest in the superficial layers. This effect was similar at both levels of the cord. On the other hand, the pattern of suppression in the lumbar spinal cord of 14 day old pups was different. At 14 days of age, in the lumbar spinal cord, Fos labeled nuclei found in the superficial layer were the most resistant to the effects of morphine.

DISCUSSION

Behavioral responses to the formalin versus thermal test

The results demonstrate that morphine administered directly into the brain produced two developmental profiles of analgesia that were distinct for supraspinally and spinally mediated behaviors; morphine administered intracerebroventrically was more effective in eliminating pain responses to formalin at an earlier age compared to the limb withdrawal from a noxious thermal stimulus. These findings are consistent with other developmental studies that have shown that

morphine blocked supraspinally mediated behaviors such as the responses following the formalin test (Guy and Abbott, 1992) at an earlier age compared with spinally mediated behaviors such as the tail flick or the hindlimb withdrawal from intense heat (Barr et al., 1992; Giordano and Barr, 1987). These results suggest that the underlying mechanisms by which morphine blocks suprapinally and spinally mediated behaviors are distinct and likely reflect developmental changes in pain processing and pain modulation in these animals. Different neurotransmitter systems are involved in processing different types of noxious stimuli in both infant and adult animal (Kuraishi et al., 1983; 1985; Giordano and Barr, 1988; Hughes and Barr, 1988). Analgesia against a noxious thermal stimulus has been correlated with inputs from 5-HT fibers. As previously suggested, the absence of analgesia in the thermal test may be due to lack of descending serotonergic inputs at more caudal levels of the spinal cord of the developing animal. Although mechanisms by which morphine blocks behavioral responses to formalin are not well described, the results of the present experiments suggest that they are mature early during postnatal life, unlike in the thermal test.

Reduction of Fos labeled nuclei

Intracerebroventrically administered morphine reduced the number of Fos immunoreactive neurons in both the thermal and formalin test at the two ages and at different levels of the spinal cord tested. Morphine administered directly into the brain can affect activities at the spinal level only through the descending control system. Electrophysiological studies have shown that the bulbo-spinal descending pathways did not influence activities at the lumbar spinal cord level until postnatal

day between 10 - 12 (Fitzgerald and Gibson, 1987). Our results suggest that the descending inhibitory pathway from the brainstem is sufficiently mature to modulate activities at the spinal level earlier than it had been previously thought. However, the results of the present study consistently showed that morphine reduced Fos immunoreactivity in the cervical level to a greater extent than the lumbar level for both formalin and thermal stimuli, suggesting greater morphine sensitivity at more rostral levels of the spinal cord. These results reflect that rostral spinal cord matures early, a finding consistent with the general rostral to caudal development of the central nervous system.

Discrepancy between behavior and Fos immunoreactivity

The results of the experiments also show that there is a discrepancy between behavior and Fos immunoreactivity. The discrepancy is more dramatic following the hindpaw stimulation, and is different for the two stimulus types. The effects of morphine on Fos immunoreactivity induced by formalin more closely resembled the adult data. In the adult, it has been consistently reported that morphine does not completely eliminate Fos stained nuclei in the spinal cord even at morphine doses that produce profound analgesia (Presley et al., 1990; Gogas et al., 1991; Jasmin et al., 1994). In the present experiment, as experiments in the adult animal, morphine eliminated behavioral responses to formalin, but it did not completely eliminate Fos immunoreactivity. It has been demonstrated that some of the morphine resistant cells that express Fos project to the parabrachial region in the caudal midbrain. However, it is still unclear why spinal cord neurons continue to express the Fos protein when the animals are not responding behaviorally to noxious stimulation.

Moreover, the functional significance of these cells projecting to the parabrachial regions is unclear. It is not known whether cells that continue to express the Fos protein also project to the parabrachial region of the brain in the immature animal. In the mature animal, itwas speculated that some nociceptive information may have been transmitted rostrally, but this was not sufficient to elicit pain responses (Jasmin et al., 1994). Another possible explanation is that nociception was blocked, but the persistent Fos staining represented molecular changes produced by injury. It is possible to make similar speculations for the immature system, but whether these speculations are indeed true need to be investigated further.

Animals tested with the thermal stimulus showed a different pattern of discrepancies between behavior and Fos immunoreactivity. Morphine pretreatment did not produce analgesia in the hindpaw at either age tested, but it eliminated immunoreactivity in the lumbar spinal cord. It is speculated that intense heat may activate two populations of spinal cord neurons; one population is opiate sensitive and shows a dose-dependent Fos inhibition following morphine administration. The other population of cells do not express Fos, and are not affected by morphine. The neurons that do not express Fos may be responsible for the transmission of noxious information and the occurrence of the withdrawal response. Because limb withdrawal from intense heat is spinally mediated behavior, these neurons may be a part of reflex arc. There is no evidence to suggest that mechanisms of analgesia at different segmental levels are distinct. If this is the case, then mechanisms by which behavioral analgesia and Fos suppression occur in morphine treatment should be similar for the forepaw and hindpaw. Analgesia was observed in the forepaw following intense heat, and the pattern of Fos suppression in the cervical cord was

strikingly similar to that observed in the lumbar cord. Despite this similarity, behaviors observed following stimulation of the two limbs were different. It is not clear why such discrepancies were observed in the infant rat. However, because the lumbar spinal cord lacks the serotonergic input in the younger animal, it is possible to speculate that serotonergic input may be required to inhibit the population of these neurons that did not express Fos and behavioral analgesia may be observed following the inhibition of these neurons as in the hindpaw.

Pattern of Fos suppression in different laminae

With an exception of thermally stimulated hindpaw of 14 day old animals, the degree of suppression in different laminae was similar. However, in the adult animal, it has been consistently reported that morphine effects in the spinal cord neurons are lamina specific (Tolle et al., 1990; Presley et al., 1990; Jasmin et al., 1994). Morphine reduces the number of Fos immunoreactive cells in the deeper dorsal horn and ventral horn to a greater extent than those in the superficial layers. Peripheral or central morphine administration prior to the application of formalin or noxious thermal stimulus inhibits Fos protein in the neck of the dorsal horn and ventral cord by 80%, but only by 60% in the superficial laminae (Gogas et al., 1992; Presley et al., 1990; Jasmin et al., 1994; Tolle et al., 1990). The adult pattern of inhibition of Fos immunoreactivity may be a function of the stimulus type. In the thermal test, the pattern of suppression of Fos immunoreactivity in the lumbar cord of the 14 day old rat began to resemble those observed in the adult rat.

In conclusion, morphine administered centrally blocks behavioral responses to formalin more effectively compared to responses from intense heat. These differences likely reflect differences in underlying mechanisms by which morphine induces analgesia in the infant rat. Despite the immaturity of the descending system. morphine administered intracerebroventrically reduces the number of Fos positive neurons in the spinal cord. but the reduction of Fos immunoreactivity in spinal cord neurons is not sufficient to produce behavioral analgesia against intense heat in the infant rat.

CHAPTER 6:

General Discussion

The first part of this thesis tracked behavioral and functional changes in rat's responses to nociception during late prenatal and early postnatal periods. The up-regulation of the c-*fos* gene was used to monitor the activity of neurons following noxious signals. Behavioral responses to tonic noxious stimuli and the functional properties of spinal neurons in pre-and-postnatal animals are included in this thesis. To study the mechanisms of antinociception, the second part of the thesis examined the effects of various agents that dampen activities at the spinal cord level in the developing system.

The results of these experiments are unique in that functional properties of a large population of spinal neurons and behavioral correlates of these activities following nociception and antinociception were monitored in the same animal.

Behavioral Responses to Formalin

Although it has been reported that one day old rat pups respond to formalin injection (Guy and Abbott, 1992; McLaughlin et al., 1990), there has been no published description of the effects of tonic noxious inputs administered in the forepaw or hindapw of the fetal rat. Fetal day 19 animals showed few responses following formalin, and the behaviors consisted of global diffuse increases in body movements. By fetal day 20, responses were more specific and included, body curls and twitches. At fetal day 21, fetuses showed well coordinated behaviors such as body curls, body twitches, leg movements, mouth opening, and face wiping. Further, fetal day 21 animals responded to the forepaw, but not to hindpaw stimulation, with face wiping and mouth opening. These results indicate that fetal

animals respond to nociceptive stimulation but that their response patterns differ from those of postnatal and adults. Neonatal animals as young as 0 day of age exhibited more directed responses to formalin including flexing of the injected paw. Three-day-old pups consistently showed lifting of the injured paw, and by 14 days of age, animals exhibited both paw lifting and licking.

In the adult, formalin injection into the paw produces a biphasic behavioral response. The first phase occurs 5 to 10 minutes after the injection and includes elevation, licking and shaking of the injected paw. These initial responses are followed by a 5 to 15 minute period without responding. Following this quiescent period, the animal licks, shakes and guards the injured paw and the responses gradually subside over the next hour or so (Franklin and Abbott, 1989). The first phase is due to chemical stimulation of nerve endings, and the second phase is thought to be a result of the peripheral inflammatory mediators such as prostaglandins and bradykinin activating C-fibers and dorsal horn nociceptive cells. However, I observed no signs of the second phase of the formalin test in the immature animal, a result consistent with a study that reported animals younger than 15 days of age exhibit no biphasic responses to formalin injection (Guy and Abbott, 1992; Barr, 1998). These data suggest that fetal and preweanling rats are processing pain and are responding behaviorally to formalin, but they have immature nociceptive systems that continue to change.

Age related changes in Fos immunoreactivity

Fos expression following formalin in prenatal animals

When fetal day (FD) 19 animals were injected with formalin, very few cells showed Fos immunoreactivity in either cervical and lumbar spinal cord. At fetal age 20, the number of Fos stained nuclei first appeared in significant numbers in the superficial lamina. The appearance of Fos in the deeper lamina was only observed in the cervical segment, and not in the lumbar level of the spinal cord. One day before birth, at FD 21, the number of Fos stained cells in the superficial and deeper lamina increased further in the cervical segement. In the lumbar segment even at FD 21 no Fos protein was expressed in the deeper lamina although there were increases in the superficial lamina. Therefore, there was a significantly greater number of Fos stained nuclei in the cervical than in the lumbar spinal cord both in the superificial and in the deeper lamina. In addition to the general increase in Fos immunoreactivity with increasing age, there were differences in the rate of increase in Fos labeled nuclei between specific ages. For example, there were large increases in Fos labeling between fetal age 20 and 21 in both cervical and lumbar spinal cord.

The pain pathway undergoes dynamic anatomical, chemical and functional changes prenatally and continues to change postnatally. The results of this thesis show that Fos results correlate particularly well with the anatomical changes that occur during late fetal life in the dorsal horn. For example, the increases in Fos immunoreactivity that are observed with increasing age in the superficial lamina may reflect the maturation of projection and interneurons as well as maturation of nociceptors. It has been shown that neurogenesis of both projection and

interneurons in the superficial lamina is completed by FD 16 (Nornes and Das, 1974), and axo-dendritic growth of the interneurons is not initiated until FD 21(Bicknell and Beal, 1984). Nociceptors that are found in the superficial lamina at FD 19.5 (Fitzgerald, 1987a) also continue to mature postnatally. The appearance of Fos stained nuclei in the deeper lamina at FD 20 is difficult to correlate with changes occurring during this time. The increase in the deeper lamina seen starting at FD 20 may be due to combination of maturation of nociceptors and projection neurons, but there are no available data to support this speculation. The absence of Fos labeling in the deeper lamina in the lumbar cord, but not in the cervical segment suggests that cells in the lumbar cord are less mature than those in the cervical cord. These conclusions are consistent with the direction of neurogenesis of the central nervous system: neurogenesis in the spinal cord follows the development in the rostrocaudal direction, so that the neurons in the cervical segment of the cord are born first followed by neurons in the thoracic and lumbar regions (Nornes and Das, 1974).

Another age related change in Fos immunoreactivity is the constituitive expression of bilateral Fos staining in areas that approximately correspond to laminae III in prenatal animals. In the stimulated fetal animal, there was an increase in the upregulation of c-*fos* gene in addition to the constitutive Fos expression. The bilateral Fos staining, which was symmetrical in the number of stained nuclei and density of the labeling, was not observed in the postnatal animals. Therefore, the transient Fos expression may reflect the maturation process rather than in transmitting noxious information, and may be due to spontaneous activity of dorsal root ganglions that is present in the late fetal life (Fitzgerald, 1987).

Fos expression following noxious stimuli in postnatal animals

Noxious thermal, chemical and mechanical stimuli were used to elicit painful signals during first two weeks of rat's postnatal life. The patterns of Fos immunoreactivity following noxious thermal and formalin are similar whereas the expression of Fos following the mechanical stimulus was distinct.

Induction of Fos immunoreactivity in the second order neurons suggests that nociceptors are capable of transmitting noxious information to the spinal cord cells and these neurons are mature enough to express Fos immunoreactivity. However, in the developing nervous system, both nociceptors and spinal cord neurons undergo changes during prenatal and early postnatal life. Therefore, changes observed in Fos immunoreactivity can be due to changes in both nociceptors and dorsal horn cells or nociceptors or dorsal horn cells alone. The expression of Fos immunoreactivity on the day of birth by mechanical, thermal and formalin suggests that the nociceptors, Aδ and polymodal C-fibers, can be activiated by these stimuli and are able to transmit noxious information to the second order neurons. This in turn suggests that these cells are able to express Fos immunoreactivity.

Thermal and formalin injection elicited Fos immunoreactivity on the day of birth and the number and the topography of Fos stained nuclei did not change during 0, 1, and 2 days of age. However, there was a large increase in the number of Fos stained nuclei between 2 and 3 days of age following both thermal and formalin stimulation with a further increase occurring between 3 and 14 days of age. At 3 days of age, most of the increase observed occurred in the superficial lamina, but following the formalin injection, there was an increase in the number of Fos labeled

cells in the deeper lamina that was not seen in thermally stimulated animals. At 14 days of age the number of cells expressing Fos continued to increase, particularly in the deeper lamina of the dorsal horn and in the ventral horn. In contrast, following thermal stimulation, the increase in the number of Fos labeled nuclei is limited largely to the superficial lamina at 14 days of age. Following the mechanical stimulus, animals expressed the highest number of Fos stained nuclei on the day of birth, and there was a significant reduction in the number of cells expressing Fos until postnatal day 2. At 3 days of age, the number of Fos positive cells began to increase with a further increase in 14 day olds. However, even in the 14 day old animals, the number of Fos labeled nuclei did not reach that observed in the newborn. Unlike pups stimulated with thermal or formalin, mechanically stimulated animals exhibited Fos labeled nuclei in the deeper lamina and in ventral horn cells on the day of birth, and this was the only age at which these cells expressed Fos immunoreactivity following mechanical stimulation.

The large number of Fos labeled nuclei found in the 0 day old pups following mechanical stimulation may reflect differential maturity of the nociceptors. It has been shown that Aδ fibers become functional earlier than polymodal C-fibers (Fizgerald and Gibson, 1984). Therefore the large number of Fos labeled nuclei found following mechanical stimulation on the day of birth may suggest that mechanical stimulation involves a greater Aδ fiber activation, whereas noxious thermal and formalin may activate only polymodal C-fibers which are less mature on the day of birth. However, the reduction in the number of Fos labeled nuclei following mechanical stimulation in 1 and 2 day old is more difficult to explain. Jennings and Fitzgerald (1996) have reported that following noxious mechanical

stimulation, the number of Fos labeled nuclei decreased with increasing age and suggested that this reduction reflects the decrease in cell density in lamina I and II with age. Those results are inconsistent with the data from this thesis. However, there are a number of important differences that may account for the inconsistency. Jennings and Fitzgerald (1996) used anesthetized pups whereas our pups were fully awake. Animals were stimulated for longer duration (10 minutes) and a more intense stimulus was used for older animals in Jennings and Fitzgerald's (1996) study. Our animals, in contrast, were stimulated for 2 or 5 (for 14 day old) seconds. All these factors have shown to affect the number, pattern and intensity of Fos labeling (Bullitt, 1990; Presley et al., 1990). The decrease in Fos immunorectivity observed between 1 and 2 day old pups may be simply due to the mechanical maturation of the paw, such as paw size, and skin thickness. This becomes particularly important issue because the intensity of mechanical stimulus was not increased for the older pup. Therefore, it is also possible that fewer Aδ fibers are activated in 1 and 2 day old pups because the stimulus may have been experienced as less painful due to the increase in the paw size in the older animals.

For all three stimulus types, there was an increase in the number of Fos labeled nuclei between the ages 2 and 3. The increase in Fos immunoreactivity occurred without changing the stimulus intensity. Therefore, it is likely that the increase in Fos labeling observed between 2 and 3 days of age may reflect changes taking place in the nociceptors or cells in the spinal cord. It is possible that this increase may reflect maturation of C-fibers where these fibers are more efficient in inducing the upregulation of Fos expression in the spinal cord neurons. Theres are no available data to support this hypothesis.

Topography of upregulation of c-fos gene in the spinal cord

In the adult, Fos staining observed in different lamina in the spinal cord following noxious stimulation has been used to suggest the cells that respond to nociception. Fos labeling has been observed predominately in the superficial lamina, with cells in the deeper layers of the dorsal horn and cells in the ventral gray expressing Fos immunoreactivity to a lesser degree in the adult.

In the immature animal, following noxious stimulus, the up-regulation of Fos immunoreactivity was consistently found in the superficial lamina even in fetal animals. However, Fos expression in the deeper lamina and ventral horn cells was most consistently observed in older animals or following intense stimulation. There are no direct projections of nociceptors to the ventral horn, and Fos immunoreactive cells observed in these lamina are polysynaptically activated. Mechanically stimulated pups exhibited Fos postive cells in the ventral horn on the day of birth suggesting that polysynaptic connections are in place as in the adult even in the 0 day old pups. The age at which deeper dorsal horn and ventral horn cells show Fos immunorectivity changed as a function stimulus types. This probably reflects intensity difference among these stimulus types rather that differential activation of spinal cord cells. For example, when the intensity of stimulus type was increased in the 3 day old pups by increasing formalin injection volumes, a greater number of cells in the deeper lamina expressed Fos immunoreactivity with higher formalin injection volumes.

Effects of different anesthetics on behavior and Fos immunoreactivity

Animals treated with ketamine-xylazine mixture and hypothermia showed signs of profound anesthesia, whereas acepromazine and methoxyflurane treated pups seemed less affected, and none of the treated pups showed any responses to the formalin injection. Despite the absence of responses to the formalin injection, when Fos expression was examined in the spinal cord neurons, different pharmacological manipulations produced different degrees of reduction in Fos immunoreactivity. Hypothermia and methoxyflurane reduced the number of Fos immunoreactive cells in the superficial layers by 83.8% and 88.6% of the control animals, respectively. Fos immunoreactivity in the deeper layers and the ventral horn was completely inhibited. Ketamine-xylazine mixture was ineffective in suppressing Fos expression. These results suggest some possible mechanisms by which these drugs produced anesthesia in young pups. Although both hypothermia and methoxyflurane produces anesthesia by suppressing activity at the spinal cord level, the exact mechanisms by which these produce anesthesia may be different. It has been shown that hypothermia blocks sensory conduction by cooling nerve fibers (Gasser, 1931). On the other hand it has been reported that volatile and gaseous agents markedly depressed excitatory postsynaptic potentials recorded in the ventral root (de Jong et al., 1968). It has been further shown that these agents do not affect the activity of primary affenents but that they act on neurons within specific laminae of the spinal cord to produce anasthesia (Heavner, 1975). Therefore, methoxyflurane probably acts directly on the spinal cord cells. Both xylazine and ketamine have shown to block activities of the dorsal horn cells in the adult (Kendig

at al., 1991; Kyles et al., 1993; Savola et al., 1991; Kitahata, Taub and Kosak, 1973; Conseiller, Benoist and Hamann, 1972). However, these agents did not suppress the Fos activity following formalin injection in the neonates. It can only be postulated that the pharmacodynamic mechanism of ketamine and xylazine may differ in the immature rat pup, so that ketamine-xylazine may exert its effects mainly in the brain sites such as thalamus and medullary reticular activiating system (Collins, 1993a).

Effects of intracerebroventrical administration of morphine on behavior and Fos immunoreactivity

The last experiment had two goals: 1) to examine if there is a difference in developmental profile of analgesia of supraspinally and spinally mediated behavior, and 2) to determine the functional maturity of the descending inhibitory system.

Behavior. Intracerebroventrical injection of morphine attenuated responses to formalin in the forepaw and hindpaw at 3 and 14 days of age, but morphine reduced pain scores to a greater extent in the forepaw. Following the thermal test, morphine increased withdrawal latencies only in the forepaw at both ages; analgesia was not observed in the hindpaw at either ages tested. It has been previously reported that intraventricular administration of morphine induced analgesia against the mechanical stimulus in the hindpaw of 14 day old rats, but not against noxious thermal stimluation at this age (Giordano and Barr, 1985; Barr et al., 1992). The apparent difference in analgesia between mechanical and thermal stimuli has been attributed to different mechanisms by which morphine attenuates pain produced by different stimulus types. In both the adult and immature animal,

noradrenergic and serotonergic systems have been implicated in producing analgesia against mechanical and thermal stimuli, respectively (Kuraishi, et al., 1985; Giordano and Barr, 1985; Tive and Barr, 1992). It has been reported that norepinephrine fibers are found uniformily in the spinal cord by PD 10, whereas serotonergic fibers are observed later (Bregman, 1987). More recently, it has been shown that processing of pain produced by the formalin test is modulated by either norepinephrine or serotonin in the adult (Liu et al, 1997). It is possible that the presence of analgesia in the hindpaw following the formalin test, but not in thermally stimulated animal reflects differential maturity of these neurotransmitter systems in the developing animal.

The first two experiments of this thesis have shown that both formalin and noxious thermal stimuli induce Fos expression in the spinal cord of the developing animal. Examining what happens to this Fos immunoreactivity in the spinal cord allowed us to more directly study how morphine adiministered into the brain can affect events occuring at the spinal level. Because morphine is administered directly into the brain, Fos expression at the spinal level can be affected only through the descending control that is known to be immature in the developing animal.

Fos expression. In general, Fos expression in the cervical cord was reduced to a greater extent by the ICV morphine following the formalin test. The pattern of Fos suppression following the formalin test suggests the immaturity of the descending control at more caudal level of the spinal cord, consistent with other studies (Fitzgerald and Gibson, 1987; Bregman, 1987). However, thermally stimulated animals exhibited no difference between the cervical and lumbar spinal cord. It may

be possible that in the thermally stimulated animal, there were simply fewer cells to suppress therefore masking the differential maturity of the descending control system at different spinal cord level. Overall, thermal stimulus produced fewer Fos labeled nuclei than did the formalin test. These results suggest that the developmental profile of analgesia is dependent on the type of stimulus used to elicit noxious input. Despite this difference between the two stimulus types used, ICV morphine suppressing Fos immunoreactivity at the spinal cord level suggests that the suppression of Fos expression does not require fully mature descending systems.

Taken together, in the developing central nervous system, at least some of the neurons showing the upregulation of c-*fos* gene following noxious stimulation are involved in transmitting noxious information because most of these neurons are modulated by anesthetic or analgesic agents. Morphine administered intracerebroventrically eliminated Fos immunoreactivity effectively in the spinal cord neurons, suggesting that the mechanisms by which morphine produces its analgesic effects such as descending inhibitory control system are immature early in the postnatal period.

Discrepancy between Fos labeling and Behavior

Results from adult studies have well documented that the number of Fos stained nuclei and the amount of pain-like behavior exhibited by the animal following formalin injection are positively correlated (Gogas et al., 1991), suggesting that Fos immunoreactivity can be used as a predictor of the amount of pain an animal is experiencing. It also suggests that Fos positive neurons in the spinal cord are

involved in pain processing. However, following both systemic and central morphine administration, despite the fact that there is complete behavioral analgesia, the expression of c-*fos* gene is not totally suppressed. Neurons in the superficial layers are most resistant to morphine effects and the inhibition never exceeds 60%. Fos immunoreactivity in the reticulated area of the dorsal horn and in the ventral horn was profoundly inhibited, with very few neurons continuing to express the protein (Presley et al.,1990). It has been shown that some of the morphine resistant Fos expressing neurons in the superficial laminae are spinoparabrachial projection neurons (Jasmin et al., 1994). The significance of c-*fos* expression during morphine analgesia is unclear.

In the immature animal, there were two types of inconsistencies between behavior and Fos staining following ICV morphine administration: in the formalin test, rats that were behaviorally analgesic continued to express Fos immunoreactivity, whereas in the thermal test, Fos expression was inhibited in rats that were not behaviorally analgesic. For example, pups tested with the thermal stimulus did not show analgesia in the hindpaw at either age tested following the morphine treatment, but therewas a marked reduction of Fos immunoreactivity.

Suppression of Fos expression was incomplete in pups that were given anesthetics and morphine.These results are consistent with the adult study (Jasmin et al., 1994; Presley et al., 1990; Gogas et al., 1989) . As suggested by Jasmin and colleagues (1994), it is possible that neurons that express Fos immunoreactivity following morphine administration are transmitting noxious signals rostrally, but activities in these cells are not sufficient to produce behaviors related to pain.

However, the suppression of Fos immunoreactivity in the absence of analgesia is more puzzling. It is possible that behavioral analgesia in the hindpaw following thermal stimulation requires inputs from a serotonergic system that is not completely mature until PD 21 (Bregman, 1987). It is also possible that in the immature system that there are spinal cord neurons that do not express the c-*fos* gene in response to noxious thermal stimulation, but nonetheless transmit noxious signal.

In summary, as reported in the adult animal, there were discrepancies between behavior and Fos. However, these discrepancies are different in the infant rat. These results suggest that 1) the discrepancy between Fos expression and behavior exists both during pain processing and dampening. 2) Fos expression is not a good predictor of behavioral analgesia, and 3) discrepancy between Fos immunoreactivity and behavior is exaggerated depending on the type of noxious stimulus.

Important Limitations of Fos as a marker for Neuronal Activity

This thesis examined the ontogeny of noiception and antinociception using Fos immunreactivity. Fos expression has been and continue to be used as a technique to map a large population of neurons that are activivated. As with any technique, there are important limitations of Fos and they are addressed below.

Significance of upregulation of the gene is unclear. One of the primary issues in using Fos in pain research, in particular, and other systems in general, is the role of functional significance of c-*fos* gene induction in these systems. There are many

immediate early genes that are activated following noxious inputs. Electrical stimulation of the sciatic nerve at Ad and C-fibers intensity induced a variety of IEG's including, c-jun, Jun B, Jun D, c-*fos*, Fos B, and Krox-24 (Herdegen et al., 1991). C-*fos* has been used most extensively due to a tight temporal link between stimulus presentation and induction of this gene, and low basal levels compared to other IEGs where c-*fos* gene mRNA can be located in the cytoplasm within minutes following stimulation (Morgan and Curran, 1989. The functional significance and differential contribution of different IEG's in pain circuitry are not known neither in the adult nor in the neonate. Naranjo and colleagues (1991) reported that neurons expressing c-*fos* gene also showed upregulation of the prodynorphin gene following thermal noxious stimulation in the hindpaw. The prodynorphin gene encodes opioid peptides that are found widely in the central nervous system including the spinal cord. The significance of the increase in the dynorphin peptide level following nociceptive input is not clear. Both antinociceptive and pronociceptive roles have been attributed to the upregulation of the dynorphin gene. These data do not demonstrate unequivocally the role that c-*fos* nor dynorphin plays in the pain pathways. The data merely suggest that induction of c-*fos* gene is correlated with increases in dynorphin peptide levels. Recently, it has been reported that c-*fos* may indeed be targeting one of the opioid genes in the spinal cord (Hunter et al., 1995; Hou et al., 1997). Intrathecal injection of c-*fos* antisense oligodeoxynucleotide blocked the translation of mRNA to protein, and thus no Fos protein was observed. Further, no increases in preprodynorphin mRNA was observed, suggesting that expression of prodynorphin gene requires the presence of Fos protein. These data suggest that there is now a growing body of evidence that

c-*fos* gene induction may have a functional role in the processing and modulation of nociception in the adult. However, the nature of that contribution and the mechanisms which mediate it are far from clear.

Negative results are not easily interpretable. The dorsal root ganglion (DRG) cells have been used as a prime example of a neuronal type that is incapable of expressing c-*fos* gene following stimulation. Other systems such as chemically induced seizure studies have shown that there is a mismatch between Fos stimulation and other markers such as the 2-DG uptake in certain brain regions (Ben-Ari et al., 1981). Metrazol seizures induce 2-DG uptake in the cerebellum and substantia nigra (Ben-Ari et al., 1981). However, metrazol seizures do not induce Fos protein in these areas. In fact, substantia nigra neurons never express Fos regardless of the types of stimuli used. It cannot be concluded from negative results that certain neuronal types never express the gene; it may well be that the circumstances or conditions under which the neuron can express the gene may have not been fully explored.

Analysis of the intensity of stained nuclei It has been consistently reported in the literature that there are intensity differences in Fos staining in both the adult and neonate (Presley et al., 1990; Williams et al., 1990). Morphine pretreatment reduces the number of Fos labeled nuclei and decreases the intensity of the labeling in neurons that continue to express the protein. These results suggest that the intensity of staining is related to the amount of stimulation a neuron receives; the darkly stained neuron is stimulated more than is a lightly labeled neuron. However, the differences observed in the intensity of Fos staining are not easily quantitated.

Although differences in intensity of labeling are recognized, majority of studies including my own have ignored the different intensity of the staining.

The results of this thesis demonstrate that fetal animals are capable of processing painful information, and during postnatal period pain processing undergoes significant maturation. Further, painful signals can be modulated by different phamacological manipulations, and in particular, morphine produces its analgesic effects by activating the descending inhibitory pathway which is functioning during early postnatal period. Despite some limitations to this technique as discussed in this chapter, examining the induction of c-*fos* expression offers a complement to other techniques in studying function of spinal neurons.

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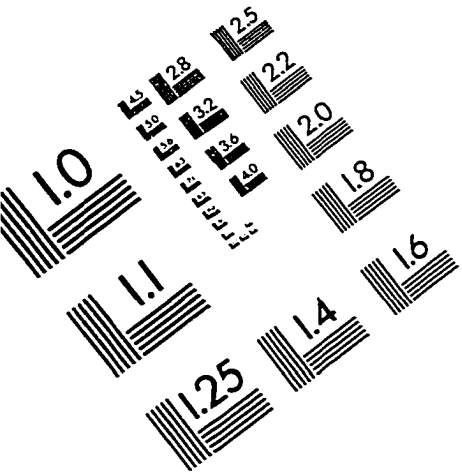
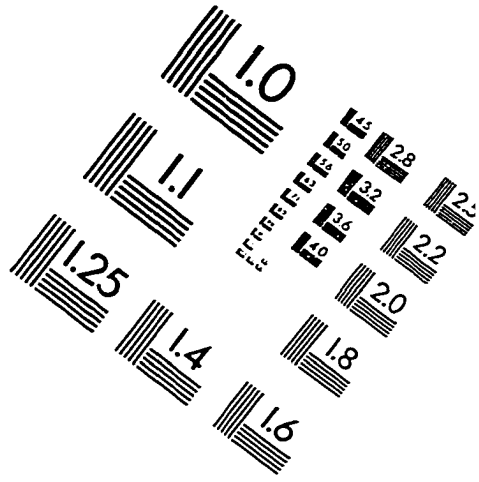
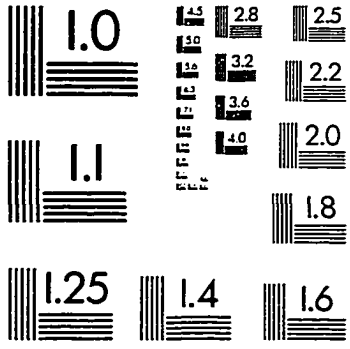
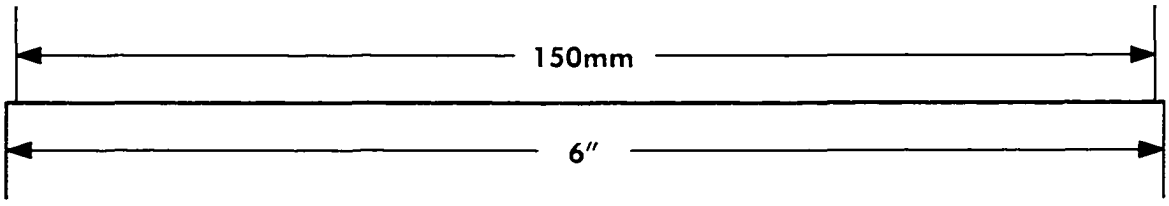
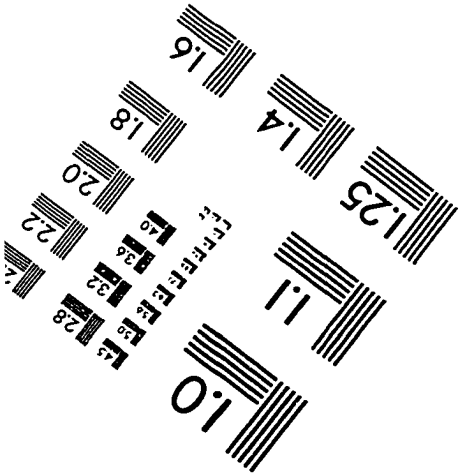
IMAGE EVALUATION TEST TARGET (QA-3)

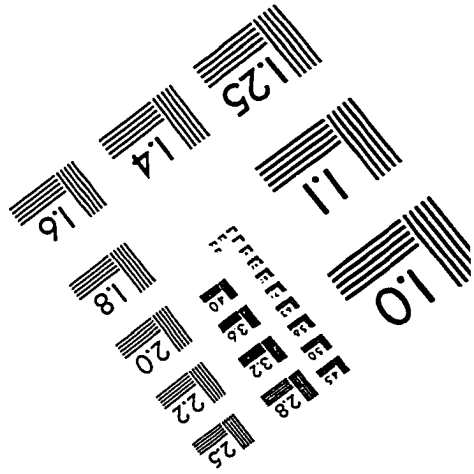












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