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PHARMACO-ONTOGENY AND POSSIBLE MECHANISMS INVOLVED IN MU- AND KAPPA-RECEPTOR MEDIATED ANTI-NOCICEPTION

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PHARMACO-ONTOGENY AND POSSIBLE MECHANISMS INVOLVED IN MU- AND KAPPA-RECEPTOR MEDIATED ANTI-NOCICEPTION

by

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Abstract

Pharmaco-ontogeny and possible mechanisms involved in mu and kappa receptor-mediated anti-nociception.

by

James Giordano

Advisor: Professor Gordon A. Barr

Both the mu agonist morphine and the kappa agonist ketocyclazocine (KC) produce analgesia in the rat. The goals of this thesis were to characterize the unique properties of mu and kappa receptor-mediated analgesia and address possible mechanisms involved in these systems.

The first set of experiments examined developmental patterns of morphine- and KC-induced analgesia in limb-withdrawal and tail-flick tests of thermal and mechanical nociception in the preweanling rat. These studies demonstrated differences in ontogeny, stimulus-specificity and somatotopy between mu and kappa opioid receptor systems. Briefly, in both thermal and mechanical tests, morphine analgesia in rostral body parts developed prior to KC-induced analgesia; while the development of KC-induced analgesia preceded morphine effects in caudal regions by several days.

Further, morphine was more analgesic in thermal and high intensity mechanical tests than KC.

Based upon these findings, a working model conceptualizing possible neural mechanisms involved in mu and kappa receptor-mediated analgesia was proposed. In this model, kappa receptors mediate analgesia segmentally within the spinal cord, local circuit effect; mu receptors, located primarily supraspinally within the midbrain periaqueductal gray (PAG) exert antinociceptive effects through interaction with descending spinal monoamine pathways. The ontogeny of kappa receptor-mediated analgesia was seen as dependent upon the extent of functional development of kappa sites in the cord, while a critical determinant of mu receptor-mediated analgesia was the rate of maturation of descending monoamine tracts.

As 5-HT has been shown to be involved in the expression of opiate analgesia, the effects of neonatal depletion of spinal 5-HT on patterns of morphine- and KC-induced analgesia were determined. Depletion of spinal 5-HT minimally affected patterns of KC-induced analgesia in both thermal and mechanical tests. Differential roles of spinal 5-HT in morphine-induced thermal and mechanical analgesia were demonstrated. Depletion of spinal 5-HT attenuated morphine-induced thermal analgesia more effectively than mechanical analgesia. These findings suggest that the raphe-spinal 5-HT system is primarily active in mu receptor-mediated thermal analgesia, and implicate the involvement of a non-5-HT brainstem system in mu receptor-mediated

mechanical analgesia. The different developmental patterns of morphine-induced analgesia against thermal and mechanical noxious input may reflect the separate maturation of these multiple PAG-bulbospinal circuits.

These studies provide further evidence that opiate drugs and endogenous opioids exert effects a several neuroanatomical loci to produce analgesia; thus, pain modulation involves activation of subpopulations of spinal and supraspinal opioid receptors as well as several bulbospinal neurotransmitter systems.

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CHAPTER ONE:

General Introduction

HISTORY AND SIGNIFICANCE OF OPIATES

The behavioral, medicinal and pharmacologic effects of opium, the dessicated sap of the poppy, Papaver somniferum, have been long known. Clinically, opiates are used in the treatment of pain and dysentery. However, opiate effects also include respiratory depression and nausea, and repeated use promotes tolerance, physiologic and psychologic dependence (Goodman & Gilman, 1982).

To understand the behavioral pharmacology of opioid receptor systems, it is important to review several of the biochemical and electrophysiological findings. A considerable body of research has addressed the biologic action of opiate drugs. In the early 20th century, Langley (1909) and later Clark (1933) proposed that many pharmacologic agents act by binding to a cellular receptor. Goldstein et al. (1971) defined the criteria and initiated the methods required for identifying an opiate-binding receptor in mouse whole brain preparations. The first demonstration of the opiate receptor came as the result of extensive independent studies by Pert & Synder (1973), Simon et al. (1973) and Terenius (1973), employing modifications of receptor binding assays in tissue homogenates. The receptor was localized to synaptosomal membranes (Pert et al., 1974) and was found both presynaptically (Atweh & Kuhar, 1977; Hiller et al., 1978) and postsynaptically (MacDonald et al., 1978; Zieglgansberger & Bayerl, 1976).

Originally, the opiate receptor was biochemically characterized as a proteolipid (Goldstein, 1977). However, solubilization techniques aimed at identifying a protein component of the receptor

have been problematic; the weak non-ionic detergents used have invariably disrupted specific binding (Simon & Hiller, 1981; Way, 1983). Subsequently, the involvements of lipids has been stressed. A role for cerebroside sulfate (CS) as the pharmacologically relevant opiate receptor has been supported by several lines of evidence: 1) CS provides an ideal site for binding of the amine nitrogen of opiates (Loh, 1977); 2) pure CS binds opiates stereospecifically (Cuatrecasas, 1977); 3) CS-deficient Jimpy mice are unresponsive to the analgesic effects of morphine (Law et al., 1978) and 4) animals treated with CS-specific antibodies are resistant to the pharmacologic actions of morphine (Craves et al., 1979). Other lipids, including phosphotidyl-serine, -phenylalanine, and -inositol have also been considered as possible functional opiate receptors (Loh et al., 1980).

Receptor-effector interactions.

Several ions and cyclic nucleotides have been suggested as effectors for opioid receptors. Divalent and monovalent cations differentially affect opiate agonist and antagonist binding: Divalent cations increase the binding affinity of agonists, while monovalent cations increase antagonist binding (Pert & Snyder, 1974). Specifically manganese, magnesium, sodium and nickel ions have been shown to enhance agonist binding (Pasternak et al., 1975; Simon et al., 1975), whereas cupric and ferric ions decrease agonist binding (Pasternak et al., 1975).

A second messenger system of cyclic nucleotides linked to opiate receptors has been well established (Blume, 1978; Childers & Snyder, 1978). Opiate receptors are negatively coupled to adenyl cyclase in a neuroblastoma hybrid cell line (Klee et al., 1978) and rat striatal slices (Childers et al., 1985). Adenyl cyclase inhibition occurs through a GTP-binding regulatory protein, Ni (Costa et al., 1985), which is GTP-ase modulated (Clark & Medzihradsky, 1985) and pH sensitive (Childers et al., 1984). As well, a cyclase independent system has been suggested (Zukin & Gintzler, 1980; Zukin & Zukin, 1984).

The involvement of the calcium-calmodulin system as a selective messenger for opiate receptors has been described by Chapman and co-workers (1980). Calmodulin is calcium sensitive, serves as a calcium binding site, and affects phosphodiesterase activity. Membrane protein kinase has been shown to be both c-AMP and calcium dependent (Clouet et al., 1981). Thus, receptor activation may elicit alterations in calmodulin, calcium and protein kinase metabolism, ultimately causing changes in membrane phosphorylation (Chapman et al., 1982).

Endogenous ligands.

The discovery of the opioid receptor prompted research aimed at demonstrating an endogenous ligand exerting opioid activity. Terenius & Wahlstrom (1974), utilizing competitive binding of opiate receptor in cerebrospinal fluid, and Hughes et al. (1975), utilizing activity of various opiates in the guinea pig ileum-myenteric plexus

preparation, independently proposed the active involvement of a low molecular weight peptide as the endogenous ligand. The Hughes group confirmed two pentapeptides, leucineenkephalin (leu-enk) and methionine-enkephalin (met-enk) as being endogenous opioids. These peptides were found to be amino acid subsequences 61-65 present in the 91 amino acid peptide beta-lipotropin (B-LPH), which had been isolated earlier by Li (1964), although its precise role had remained vague. Guillemin et al. (1976) isolated three peptides from extract of sheep hypothalamus and hypophysis. These peptides were also found to be residues of the B-LPH molecule, and were referred to as alpha-endorphin (amino acid sequence 61-76), beta-endorphin or C-fragment (amino acid sequence 61-91) and gamma-endorphin (amino acid sequence 61-79). The opioid activity of these substances was confirmed by Bradbury et al. (1976) and Cox et al. (1976).

Another peptide, dynorphin, was isolated from hypothalamus and hypophysis by Goldstein et al. (1980). Dynorphin (1-17) and its subfragments dyn (1-8/9), dyn (1-11) and dyn (1-13) have been shown to possess varying degrees of opioid activity in vitro and in vivo (Corbett et al., 1982).

Akil et al. (1976) have shown that B-LPH and the endorphin fragments exist in the same neurons, suggesting a possible biosynthetic route wherein alpha, beta and/or gamma endorphin may be enzymatically converted from the B-LPH precursor. However, Nakanishi et al. (1979) have asserted that leu- and met-enkephalin are not produced by the same bioconversions as the larger molecular weight opioids. Circulating B-LPH or beta-endorphin of hypophyseal origin

did not appear to be the source of brain enkephalin, as hypophysectomy did not affect the concentration of either leu- or met-enkephalin in brain (Hong et al., 1977; Kobayashi et al., 1978). Recently, Hughes (1983) has categorized the endogenous opioids into three groups based upon their respective precursor molecules. These are 1) the proopiomelanocortin group, which gives rise to alpha, beta and gamma endorphin, as wellas ACTH and MSH; 2) the proenkephalins, from which leu- and met-enkephalin are derived; and 3) the prodynorphin group, which is precursor to the dynorphin peptides and possibly an additional source of leu-enkephalin. A complete review is provided by Akil et al. (1984).

Multiple opiate receptors.

The differential actions of opiate drugs strongly implied the presence of heterogeneous populations of opiate receptors. Originally, Martin et al. (1976) proposed two opiate receptor sites based on agonist/antagonist activity of morphine and nalorphine. Three opiate receptor subtypes were later suggested based on actions of classes of opiates in the morphine-dependent and non-dependent chronic spinal dog (Martin et al., 1976b). These receptors were named after their principal agonists and were found to mediate specific physiologic effects. Morphine was the principal ligand for the mu receptor and caused analgesia, respiratory depression, bradycardia and euphoria. Ketocyclazocine (KC) was the principal agonist for the kappa receptor, causing analgesia, sedation and miosis. N-allylnormetazocine (SKF-10, 047), the principal sigma

agonist caused respiratory stimulation, mydriasis and was psychotomimetic. Further, it was subsequently found that the enkephalin pentapeptides were more potent than morphine in the mouse vas deferens (MVD) bioassay, while the potency of morphine was greater in the guinea pig ileum (GPI) preparation. Based upon these findings, a fourth opiate receptor, the delta site, relatively specific for D-ala-D-leu-enkephalin (DADLE) was described (Lord et al., 1977). Binding (Chang & Cuatrecasa, 1979), differential tolerance (Schulz et al., 1980, 1981), protection (Robson et al., 1979) and radioautographic studies (Duka et al., 1981; Goodman et al., 1980) have confirmed the presence of distinct delta sites. While alternative schemes of opiate receptor classification exist (see Pasternak et al., 1980; Pert et al., 1981; Quiron et al., 1982), the mu, kappa, delta classification as originally proposed by Martin and co-workers (1976a, b) and Lord et al. (1977) is favored (see Table 1).

Several studies have addressed the behavioral and pharmacologic effects mediated by the mu and kappa receptors. There is question as to whether the effects of ketocyclazocine-like opiates are mediated by a receptor which is distinct from the mu site. Although the benzomorphan drugs are capable of binding to a high affinity mu site (Pasternak, 1980), there is mounting evidence in support of a unique kappa receptor system. Selective tolerance between mu and kappa agonists has been demonstrated in GPI (Schulz et al., 1981) and MVD (Wuster et al., 1981). Oka and colleagues (1981) reported that only kappa agonists were effective in suppressing electrically induced

RECEPTOR	PRINCIPAL LIGAND	BIOLOGIC/BEHAVIORAL EFFECTS	REFERENCE
Mu (also MU_1 MU_2)	MORPHINE	ANALGESIA, EUPHORIA RESPIRATORY DEPRESSION	LORD et al (1977) MARTIN et al (1976) PASTERNAK et al (1975, 1980)
KAPPA	KETOCYCLAZOCINE-LIKE BENZOMORPHANS, U-50,488H, DYNORPHIN	SEDATION, ANALGESIA	CHAVKIN et al (1982) CORBETT et al (1982) KOSTERLITZ et al (1981)
SIGMA	N-ALLYL-NORMETAZOCINE (SKF-10,047)	DELIRIUM, DYSPHORIA, RESPIRATORY STIMULATION	ZUKIN et al (1982)
DELTA	D-ALA-D-LEU-ENKEPHALIN	ANALGESIA	Lord et al (1977)

TABLE 1

contractions of the rabbit vas deferens (RVD). Mu and delta agonists were inactive in this preparation, indicating that RVD contains only kappa receptors, and is therefore a specific kappa bioassay. Further, in both RVD and GPI, benzomorphans were less sensitive to naloxone antagonism than were mu agonists (Hutchinson et al., 1975; Oka et al., 1981). In cross-protection studies, kappa drugs were more effective than mu, or delta ligands in preventing kappa receptor alkylation by phenoxybenzamine (Kosterlitz et al., 1981). In binding studies, 3H-ethylketocyclazocine was less displaceable by unlabeled morphine or DADLE than kappa agonists (Wolozin et al., 1982).

Additional support for a distinct kappa receptor comes from findings that dynorphin peptides may be the endogenous kappa receptor ligands. Results from several research approaches strongly suggest the kappa activity of dynorphin: 1) In RVD, dyn A, dyn(1-9), dyn(1-11) and dyn(1-13) have been shown to yeild a potency series (Oka & Negishi, 1982); 2) the action of dyn(1-13) was more completely reversed by MR-2266, a kappa-specific antagonist, than by naloxone (Oka et al., 1982); 3) In GPI, dyn(1-13) completely protected kappa binding sites against alkylation by B-chlornaltrexamine (Chavkin et al., 1982); 4) The high concentration of immunoreactive dynorphin in the cord (Botticelli et al., 1982; Goldstein et al., 1982) corresponds to the distributions of spinal kappa receptors (Slater et al., 1983; Traynor et al., 1983); 5) Intrathecal administration of dyn A produced analgesic effects which were 6-10 fold more potent than intrathecally administered morphine, and this potency remained unchanged in morphine tolerant rats (Han & Xie, 1982). While Tung

and Yaksh (1982) reported that intrathecal dyn(1-13) failed to produce analgesia, these conflicting results have subsequently been attributed to methodological differences (Goldstein, 1984).

Evidence that selective mu and kappa agonists exert their effects at different neuroanatomical loci further strengthens the argument for separate mu and kappa receptor systems. Morphine administered intracerebrally to the periaqueductal gray (PAG) area produced significant and analgesia (Jacquet & Marks, 1976; Lewis & Gebhardt, 1977; Yaksh & Rudy, 1976), while injection of EKC to that area (Wood et al., 1981) or to the lateral ventricle (Wood et al., 1982) was ineffective in eliciting an analgesic response. Intrathecal administration of bremazocine, EKC or the novel kappa agonist U-50, 488H produced analgesia in the tail-flick paradigm (Piercey et al., 1984); von Voigtlander et al., 1983). Moreover, spinal transection attenuated morphine- but not EKC-induced analgesia (Dewey et al., 1969; Wood et al., 1981). These findings correlate to the anatomical distribution of mu and kappa receptors. Mu receptors have been localized to the superficial cortical layers, interpeduncular nucleus, pontine and reticular zones of the brain stem, particularly areas proximal to the locus coeruleus and midline raphe nuclei, the periaqueductal/periventricular gray regions of the midbrain and within the spinal cord (Goodman et al., 1980). Kappa receptors exhibit greater concentration in laminae V-VI of the cerebral cortex, caudal brain stem, spinal vestibular nuclei and throughout the spinal cord (Goodman & Synder, 1982; Slater & Patel, 1983; Uhl et al., 1978).

Developmental studies have illustrated differences between these receptor systems aswell. Initially, it was shown that 3H-naloxone and 3H-naltrexone binding increased with age (Clendeninn et al., 1976; Coyle et al., 1976), and had a caudal to rostral pattern of maturation in brain (Tsang et al., 1982; Tsang & Ng, 1980). Patterns of analgesia have been correlated with opiate receptor ontogeny. A high affinity binding site for morphine, naltrexone and EKC developed no earlier than 12 to 14 days after birth, and paralleled the ontogeny of morphine analgesia in the tail-flick test (Pasternak et al., 1980; Zhang & Pasternak, 1981). The development of a low affinity receptor peaked several days earlier (Pasternak et al., 1980; Zhang & Pasternak, 1981) and has been associated with the ontogeny of EKC-induced analgesia in the tail-flick paradigm (Barr et al., 1983). In the neonatal rat, kappa and delta receptors were found to mature prior to mu receptors (Kornblum et al., 1985; Leslie et al., 1982; Wohltmann et al., 1982).

In addition to developmental differences, somatotopic variations in the patterns of opioid analgesia have been described (Watkins et al., 1982a, b). Both body topography and stimulus parameters have been shown to affect the neurochemical basis of analgesia (Cannon et al., 1982, 1984). Somatotopic differences in patterns of opioid analgesia may reflect mediation of antinociception by subpopulations of opioid receptors acting at different regions within the central nervous system. It is possible that distinct opioid receptor mechanisms may be differentially engaged by the location, type and intensity of the noxious stimulus.

Opiate analgesia may be mediated in part by opioid receptors within the spinal cord (Yaksh, 1978, 1981) and by supraspinal sites influencing descending bulboreticular spinal systems (Basbaum & Fields, 1984). Several studies have described the possible organization of this neuraxis. Primary nociceptive A-delta and C fibers terminate in laminae I, IIa and V of the dorsal horn of the spinal cord (Light & Perl, 1979). The majority of second order neurons decussate in the cord, aggregate in the anterolateral columns, and ascend to higher loci, including the PAG (Kelly, 1980). Several structures of the rostro-ventral medulla (RVM), including the nucleus reticularis gigantocellularis (NRGC), nucleus reticularis paragigantocellularis (NRpG) and particularly the nucleus raphe magnus (NRM) receive projections from the PAG (Beitz et al., 1983; Carlton et al., 1983; Pomeroy et al., 1979). Fibers from these nuclei descend in the dorsal horn of the spinal cord, primarily within the dorsolateral funiculi (DLF) (Basbaum & Fields, 1979; Bowker et al., 1981; Dostrovsky et al., 1983) and synapse on neurons in laminae II and V of the dorsal horn (Ruda & Gobel, 1980). Recent neuroanatomical (Bowker et al., 1981; Johanssen et al., 1981) and physiological data (Fields et al., 1983; Gray & Dostrovsky, 1983; Pomeroy et al., 1979; Vanegas et al., 1984) have confirmed the presence of this circuit. Stimulation of the PAG activated neurons within the NRM (Morrow & Casey, 1984; Oleson et al., 1978), inhibited nocisponsive neurons within the dorsal horn (Dubuisson & Wall, 1980) and produced potent behavioral analgesia (Behbehani & Fields, 1979;

Fields & Andersen, 1978; Gray & Dostrovsky, 1983; Guilbaud et al., 1980; Willis, 1982).

Mechanisms of mu and kappa analgesia

An important unresolved issue in the study of mu and kappa receptor-mediated analgesia is the mechanisms through which opiates act to produce pain modulation. A role for serotonergic neurotransmission in both mu and kappa analgesia has been suggested. Von Voigtlander et al. (1984) have shown that suppression of 5-HT function with parachlorophenylalanine (pCPA), cyproheptadine, ketanserin or pirenperone blocked the analgesic potency of the kappa agonist U-50, 488H but not morphine to thermally noxious stimuli. Similar findings have been reported by others (Cheney et al., 1971), and indicate that kappa analgesia may be dependent upon serotonergic function. Evidence supporting serotonergic involvement in mu analgesia, however, is equally compelling. Supraspinal stimulation induced analgesia was blocked by intraperitoneal administration of the 5-HT synthesis inhibitor pCPA (Carstens et al., 1983). Lesioning the NRM attenuated morphine-induced analgesia (Azami et al., 1978). Morphine analgesia against thermally noxious stimuli was blocked by intrathecal administration of the 5-HT antagonist methysergide (Yaksh et al., 1978). Morphine analgesia was attenuated by depletion of 5-HT in the cord with the neurotoxin 5,6-dihydroxytryptamine (5,6-DHT) (Kuraishi et al., 1984; Proudfit & Yaksh, 1980). Further, Tyce & Yaksh (1981) and Yaksh & Tyce (1979) have demonstrated

increased release of 5-HT within the dorsal horn of the cord following microinjection of morphine into the PAG.

The involvement of this descending raphe-spinal 5-HT system in morphine analgesia may be dependent upon the type of noxious stimulation (Kuraishi et al., 1984). Intrathecally administered 5-HT was most analgesic in tail-flick and hot-plate tests of nociception (Kuraishi et al., 1985). Depletion of spinal 5-HT with 5,6-DHT decreased the potency of morphine analgesia against thermal pain (Kuraishi et al., 1984). These data suggest that the PAG-spinal serotonergic system selectively alters the processing of input from thermal nocisponsive units within the dorsal horn.

There is evidence to demonstrate that this raphespinal tract is immature at birth and develop postnatally. Immunoreactive 5-HT fibers developed within 3-5 days after birth in cervicothoracic areas of the cord, while immunoreactive 5-HT in lumbo-sacral cord did not markedly increase to mature levels until 15 days postnatally (DiTirro et al., 1983; Ho et al., 1981). Serotonin-like immunoreactivity in laminae I and II of the superficial dorsal horn matured earlier in cervical regions than in lumbar areas (Gilbert et al., 1979; Martin et al., 1978). These findings illustrate the caudal progression of spinal 5-HT systems in the developing animal and suggest differential serotonergic innervation of spinal targets during ontogeny.

Both morphine and ketocyclazocine produce analgesia in the preweanling rat. However, the question arises as to whether the patterns of analgesia represent a purely spinal effect, may be attributed to interaction of supraspinal receptor sites with

descending systems, or both. Data from pilot studies have shown that morphine-induced analgesia preceded ketocyclazocine-induced analgesia to thermal and mechanical noxious stimuli in the forepaws. Ketocyclazocine-induced analgesia appeared several days prior to morphine analgesia in the hindpaws and tail. I propose that these data reflect different processes involved in morphine- and ketocyclazocine-induced analgesia. One possibility is that appropriate spinal receptors may develop earlier in more rostral regions. The extent of analgesia may be mediated by the degree of caudal development of these receptors. Thus, mu receptors may appear earlier than kappa sites in the thoracic spinal cord, while kappa receptors would mature earlier than mu receptors in the lumbosacral cord. However, the development of mu-analgesia in the hindpaws and tail during the second postnatal week, in light of the apparent scarcity of mu receptor sites in the caudal cord (Gourderes et al., 1981), strongly implies a late-maturing component other than lumbosacral populations of mu receptors. While this element may be spinal delta sites (Yaksh, 1984), it is also possible that the development of descending bulbospinal systems may contribute to the delayed maturation of morphine-induced analgesia in the hindpaws and tail. Therefore, I hypothesize a model in which morphine acts primarily through supraspinal mu receptors, which exert effect over descending spinal monoamine tracts. These mu receptor sites, located within the mesencephalic PAG, might develop early, while descending monoamine pathways, assuming a rostro-caudal pattern of ontogeny in the cord, take longer to develop. The mu receptors may be

functionally active as early as 3 days after birth, however, an important determinant of analgesic effects would be the rate of maturation of the descending spinal tracts. The neural circuitry involved in mu analgesia of the forepaw may be developed and active at 3 days of age, while circuitry of more caudal structures (i.e. - hindpaws, tail) develops later. I propose that ketocyclazocine acts primarily through spinal kappa receptors. These kappa receptors may mediate anti-nociception on a segmental level. Patterns of ketocyclazocie analgesia would reflect the rostro-caudal maturation of kappa sites within the cord.

General Methods

The following experiments examined the ontogeny and mechanisms of action involved in mu and kappa opioid receptor-mediated analgesia in the rat. By characterizing the changing properties of analgesia in response to specific opiate drugs in the developing animal, I hope to further define possible neural mechanisms subserving mu and kappa analgesia, and to clarify the behavioral and physiologic roles of these opioid receptor systems.

The first experiment investigated the differential pattern of ontogeny of morphine- and ketocyclazocine-induced analgesia in the forepaw and hindpaw to a thermally noxious stimulus, and compared these findings to existing data for the tail (Barr et al., 1983). The second experiment examined the development of morphine- and ketocyclazocine-induced analgesia to mechanical noxious stimuli. In this paradigm, a high and low intensity nociceptive stimulus was used

to investigate the effects of stimulus intensity on mu and kappa opioid receptor mediated analgesia. These experiments demonstrated developmental and somatotopic differences between mu and kappa receptor mediated systems, and compared the analgesic effectiveness of these systems to different nociceptive input.

As serotonin has been shown to be involved in the expression of opiate analgesia, the third experiment determined the effects of neonatal spinal serotonergic depletion on the development of morphine and ketocyclazocine analgesia against both thermal and mechanical noxious stimuli.

These studies address the pharmacology of mu and kappa receptor systems in the developing rat. These approaches attempt to clarify the functional roles of these systems in analgesia, and hopefully will further contribute to progress in the understanding of pain and pain modulating systems.

CHAPTER TWO

Mu and kappa receptor-mediated analgesia in the developing rat: Differences due to type of noxious stimulus and body topography

INTRODUCTION

Several groups have demonstrated the existence of heterogeneous opioid receptors (Chang et al., 1978; Lord et al., 1977; Pasternak et al., 1975; Zukin et al., 1981). Evidence for distinct mu and kappa opioid receptors comes from in vitro competitive binding studies in brain homogenates (Chavkin et al., 1982; Goodman et al., 1982; McLawhon et al., 1982), cross-protection studies (James et al., 1982; Kosterlitz et al., 1981), differential antagonist reversibility (Cox et al., 1983; Kosterlitz et al., 1981) and cross-tolerance studies (Tung et al., 1982; Wuster et al., 1981). Chapter 1 provides an overview of these studies. In vitro homogenate binding and radioautographic studies have shown different distributions of mu and kappa receptors throughout the CNS mu receptors have been localized to interpeduncular nucleus, pontine and reticular zones of the brainstem, periaqueductal (PAG) and periventricular gray regions of the mesencephalon, and spinal cord (Goodman et al., 1980; Snyder, 1975). Kappa receptors have been localized to the caudal brainstem, deep cortical layers, spinal vestibular nuclei and throughout the spinal cord (Czlonkowski, 1983; Slater et al., 1983). These distributions correlate with findings that have shown that mu and kappa agonists exert their analgesic effects at different neuroanatomical loci. Morphine produced analgesia when administered intracerebrally to the PAG (Jacquet et al., 1976; Lewis et al., 1977; Wood et al., 1981; Yaksh et al., 1976), while ketocyclazocine (KC) did not (Wood et al., 1981, 1981a). Spinal transection attenuated morphine-induced, but not KC-induced analgesia (Dewey et al., 1969;

Wood et al., 1981). Although conflicting reports exist (Porrecca et al., 1984), vonVoigtlander and co-workers (1982, 1984) have shown that spinal administration of the novel kappa agonist U-50, 488H produced analgesia in tests of thermal nociception.

Opioid receptors display non-uniform ontogeny and show a caudal to rostral pattern of maturation in brain (Tsang et al., 1982; Pasternak et al., 1980). Patterns of analgesia against a thermal stimulus have been correlated with opioid receptor ontogeny; KC-induced analgesia preceded morphine-induced analgesia in tests of thermal nociception, and paralleled the earlier development of kappa receptors (Barr et al., 1983; Giordano et al., 1984). Morphine-induced analgesia in the tail-flick test appeared 7 to 13 days after birth in the rat, and was shown to parallel development of a high-affinity opioid receptor, the mu-1 site (Pasternak et al., 1980; Zhang et al., 1981). A low-affinity opioid receptor developed several days earlier (Pasternak et al., 1980).

Additionally, mu and kappa receptor-mediated analgesia may be differentially dependent upon the type and intensity of the noxious stimulus (Cannon et al., 1984; Dennis et al., 1980; Tyers, 1980). Hayes et al. 91983) and Kaneko et al. (1983) reported that kappa opiates were more effective in producing analgesia against mechanical stimuli, while morphine appeared to inhibit thermal nociception more effectively (Schmauss et al., 1984). Somatotopic differences in sensitivity to nociceptive stimuli (Cannon et al., 1984; Watkins et al., 1982) indicate the complexity of mu and kappa receptor-mediated analgesia.

The first goal of the present study was to examine the development of morphine- and KC-induced analgesia. The second goal was to characterize different developmental patterns of analgesia mediated by morphine (mu) and KC (kappa) receptor systems and to define somatotopic and stimulus specific differences in analgesia produced by morphine and KC.

MATERIALS AND METHODS

Subjects

Subjects were Long-Evans rat pups aged 3, 5, 7, 10 and 14 days, and were offspring of rats mated in our colony. Pups were maintained at constant nest temperature (33-35^{o}C) prior to testing. The number of pups used was 4-10 per dose at each age and animals were randomly assigned to treatment and control groups. Each pup was used only once; within a drug group, dose-effects (including controls) were assessed from animals from the same litter. Thus, the number of pups used per experiment refers to the number of litter groups in each experimental paradigm.

Drugs

Drugs used were morphine sulfate (generously supplied by Pennick Corporation), KC (generously supplied by Drs. Scott and Soria, Sterling Winthrop Corporation) and naloxone hydrochloride (generously supplied by DuPont Pharmaceuticals). All drugs were dissolved in distilled water and were administered in a volume of 1 ml/100g body weight.

Appropriate drug doses in each paradigm were determined in pilot experiments in which maximal analgesic dose ranges of morphine and KC were examined. These investigations demonstrated that 1.0 mg/kg was the minimal effective dose at which both morphine and KC were capable of inducing analgesia. Further, it was found that doses in excessof 10.0 mg/kg produced asymptotic analgesic effects and produced severe respiratory depressive and motor effects.

PROCEDURE

Thermal Tests

Pups aged 3, 5, 7, 10 and 14 days old (N=8 per dose x age) were administered either 0.0, 1.0, 3.0 or 10.0 mg/kg of morphine or KC intraperitoneally. All testing occurred 30 minutes post-injection. This time was determined in pilot experiments to be the time of maximal analgesic effect for both drugs. Thermal analgesia was tested by immersing the forepaw and hindpaw in random order in a bath of hot water (50^{o}C) and recording withdrawal response latencies (in 1/100 sec. intervals) on a foot-pedal operated electrical timer (Lafayette Industries, model #20225-ADW). In this paradigm, a cut-off of 5 seconds was used, after which the stimulus was discontinued.

Mechanical Tests

To test mechanical analgesia, a blunt probe, fashioned from a dulled 23 ga. needle, bevelled to an angle of 30^{o}, 0.2 cm in diameter, was applied with a force of 15 g. to the dorsal side of the

forepaw, hindpaw and tail in random order, and withdrawal response latencies were recorded. A cutoff latency of 5 seconds was used. In another experiment, a blunt probe, fashioned from a dulled 23 ga. needle bevelled to an angle of 90^{0}, 0.2 cm in diameter applied at 10 g. of force to the dorsal side of theforepaw, hindpaw and tail in random order was used to determine differences in analgesia due to decreasedstimulus intensity. Mechanical stimulus was determined by lowering the stimuli onto the surface of a balance and recording pressure weight. Within this paradigm, it is critical to maintain an operational definition of stimulus intensity. In this context, stimulus intensity refers to the properties of a specific stimulus modality (e.g. - mechanical, thermal) that elicit different withdrawal response baselines. Thus, a more intense stimulus will elecit a decreased baseline response, while a less intense stimulus will elicit an increase in response baseline. While the withdrawal topography was identifcal for both high and low intensity stimuli, the response latencies were longer when the low intesnity stimulus was applied. Response latencies were recorded both prior to and 30 minutes post-injection. A cut-off of 10 seconds was used, after which the stimulus was discontinued.

Naloxone Antagonism

Pups aged 7 and 14 days (N+8 per dose x age) were administered 10.0 mg/kg of either morphine or KC intraperitoneally. Naloxone was administered in a dose range of 0.001 mg/kg to 1.0 mg/kg. The antagonist was administered 20 minutes after the opiate agonist. Ten

minutes after naloxone injection, mechanical analgesia was assessed according to methods previously described.

All analgesic effects were evaluated by analysis of post-injection response latencies. All data were analyzed by four-way factorial analysis of variance (ANOVA). Factors were drug (morphine or KC), dose, body part (forepaw, hindpaw, tail) and age. The unit of analysis was the litter. Separate analyses were done for thermal and high and low intensity mechanical stimuli. Post-hoc comparisons of significant interactions were done by tests of simple main effects (Kirk, 1968).

RESULTS

Thermal Tests

The data demonstrating morphine and KC-induced analgesia in response to the thermal stimulus are presented in the first figure. The data on the tail-flick, (taken from Barr et al., 1983) are presented for comparison, but were not included in the analyses. The results of ANOVA for the forepaw and hindpaw showed significant differences between the two drugs ($p<.001$), between the forepaw and hindpaw ($p<.001$) and a significant interaction between the limb tested and the two drugs ($p<.001$). There were no significant age effects. The data demonstrate that when the thermal stimulus was presented to the forepaw, morphine produced analgesia in a dose-dependent manner as early as 3 days of age. Morphine was always more potent than KC against thermal stimuli in the forepaw. The effects of the drugs in the tail-flick were markedly different. KC

produced robust analgesic 2-4 days prior to morphine, with the major increase in effect occurring between 9 and 10 days of age (Barr et al., 1983). Neither drug produced significant effects in the hindpaw withdrawal test (see Figure 1).

Mechanical Tests

When the high intensity mechanical stimulus was presented to each limb, there were marked differences in results compared to data using the thermal stimulus. All main effects and interactions in the ANOVA were significant ($p<.001$, with one exception, $p<.05$). In the forepaw, morphine was more potent than KC, and the onset of analgesia for both drugs was between 3 and 7 days of age in this appendage. In the tail-flick test, in agreement with data using the thermal stimulus, KC was more effective in blocking the response in 7 and 10 day olds, but by 14 days, morphine was more effective. Analgesia developed between 5 and 7 days of age in the hindpaw as well, and morphine was slightly more effective than KC (see Figure 2).

When a low intensity mechanical stimulus was applied, and baseline latencies increased, a third pattern of results was demonstrated. All main effects and interactions were significant ($p<.001$). When the stimulus was applied to the forepaw, morphine was more effective than KC at early ages. Morphine was analgesic at 3 days of age, and both morphine and KC showed a notable increase in analgesia at 7 days postnatally. In the tail-flick test, neither drug was effective until 7 days of age, at which time KC induced potent analgesia. Morphine produced minimal effects until 14 days of

Figure 1. Effects of morphine and KC on withdrawal from a thermally noxious stimulus. Points represent mean response latencies (±SEM) to immersion of the forepaw, and hindpaws into a bath of hot water (50°C). Existing data for the tail from Barr et al. (1983) are presented for comparison.

FIGURE 1

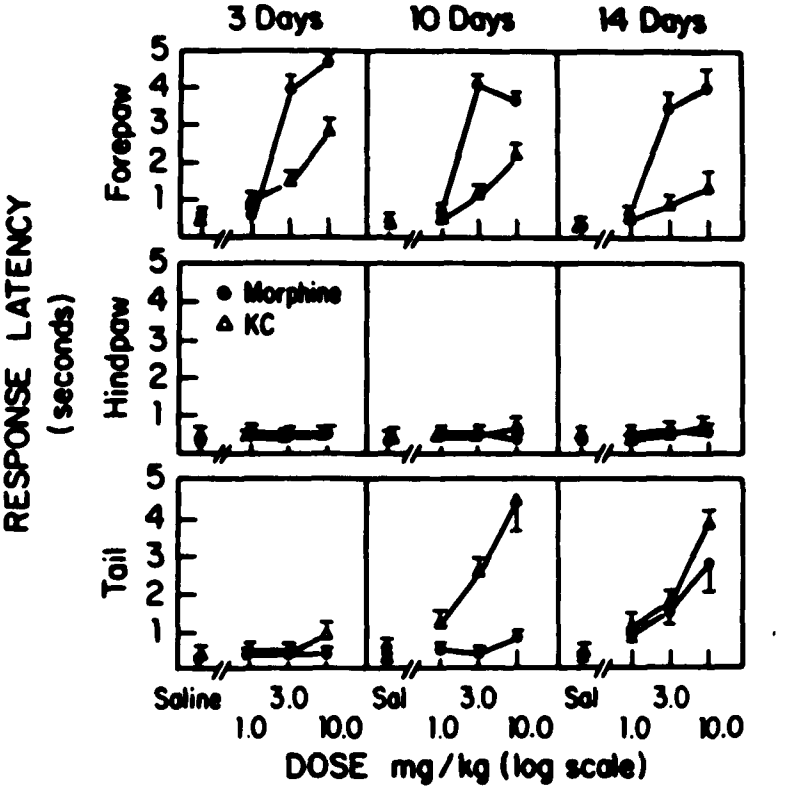
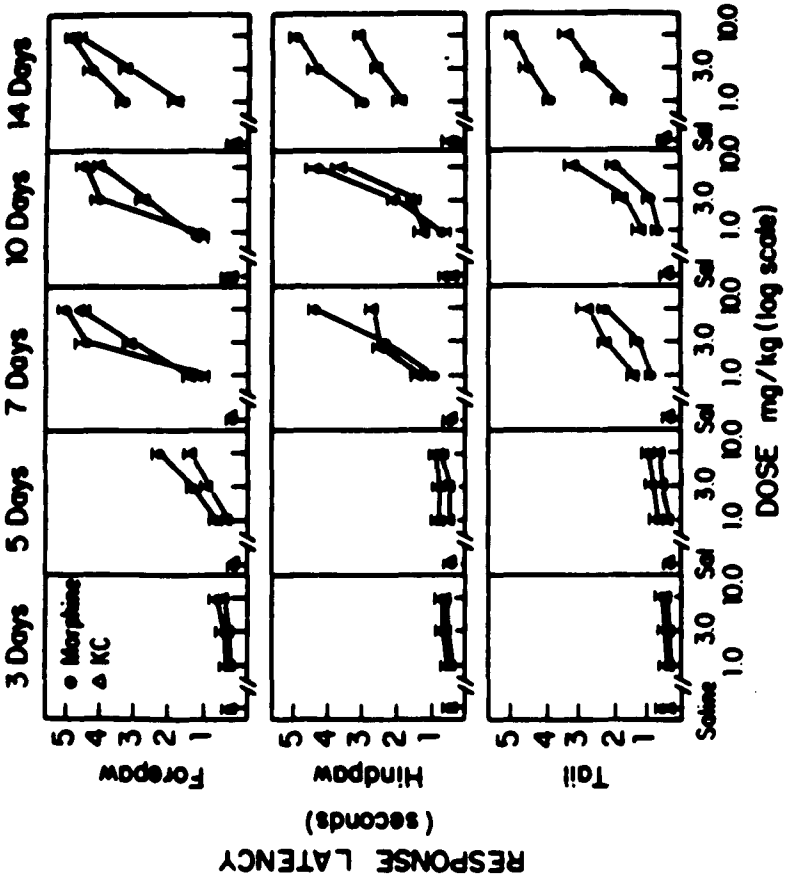

3 Days
10 Days
14 Days
RESPONSE LATENCY (seconds)
Forepaw
Hindpaw
Tail
Morphine
KC
Saline
Sal
3.0
1.0
10.0
DOSE mg/kg (log scale)

FIGURE 2. Effects of morphine and KC on withdrawal from a high intensity mechanical stimulus. Points represent mean response latencies (±SEM) to application of a 30^{0} angled probe at 15 g. of force to the dorsal side of the forepaw, hindpaw and tail.

FIGURE 2


3 Days
5 Days
7 Days
10 Days
14 Days
● Morphine
▲ KC
Forepaw
Hindpaw
Tail
RESPONSE LATENCY
(seconds)
Saline
1.0
3.0
10.0
DOSE mg/kg (log scale)

age. In the hindpaw, KC was more effective than morphine at 7 and 10 days of age. As in the tail-flick test, morphine had only marginal analgesic action until 14 days of age (see Figure 3).

Naloxone Antagonism

The data showing the effects of naloxone on morphine and KC-induced analgesia are presented in Figure 4. Both morphine- and KC-induced analgesia were naloxone reversible. A higher dose of naloxone was required to antagonize morphine-induced analgesia. There were no differences among appendages. Naloxone reversal of KC-induced analgesia was dose-dependent while full reversal of morphine-induced analgesia was seen even at the lowest dose tested. KC-induced sedative effects were reversed at lower doses than KC-induced analgesia as evidenced by qualitative measures of sedation such as postural flaccidity and catalepsy (data not presented).

DISCUSSION

Morphine was consistently more effective than KC when either thermal or mechanical stimuli were applied to the forepaw. The analgesic actions of morphine appeared between 3-5 days of age in thermal and mechanical tests of nociception. In the tail-flic test, KC was consistently more potent than morphine at younger ages. The onset of KC effects was between 7 and 10 days, depending on the stimulus. Morphine effects did not peak until 14 days of age in both thermal and mechanical analgesic tests. Against a less intense mechanical stimulus, morphine was not very effective at any age

FIGURE 3. Effects of morphine and KC on withdrawal response latencies from a low intensity mechanical stimulus. Points represent mean response latencies (±SEM) to application of a 90^{o} angled blunt probe to the dorsal side of the forepaw, hindpaw and tail.

FIGURE 3

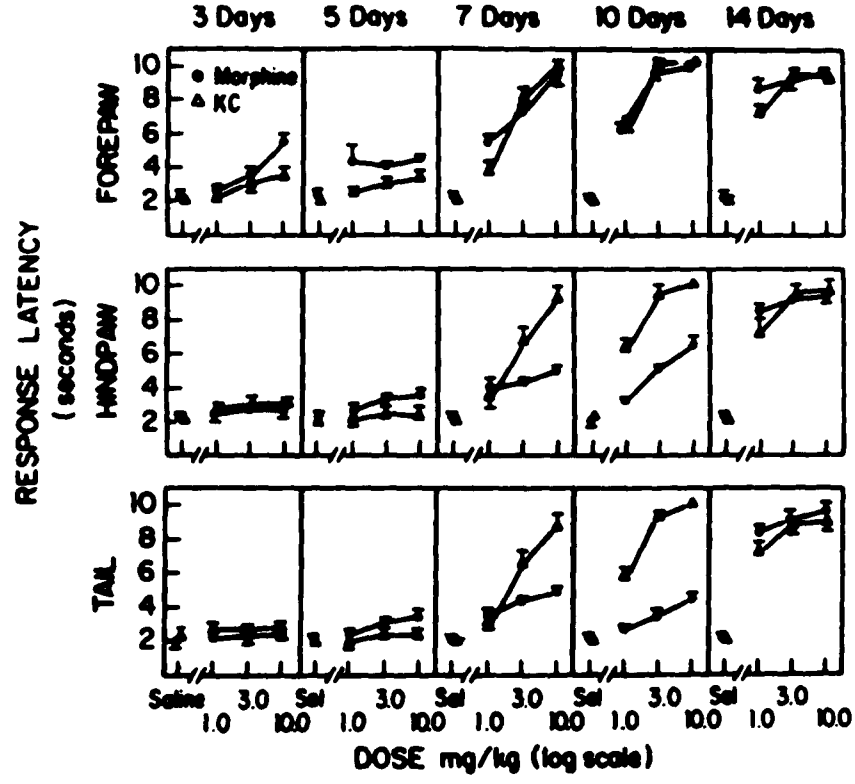
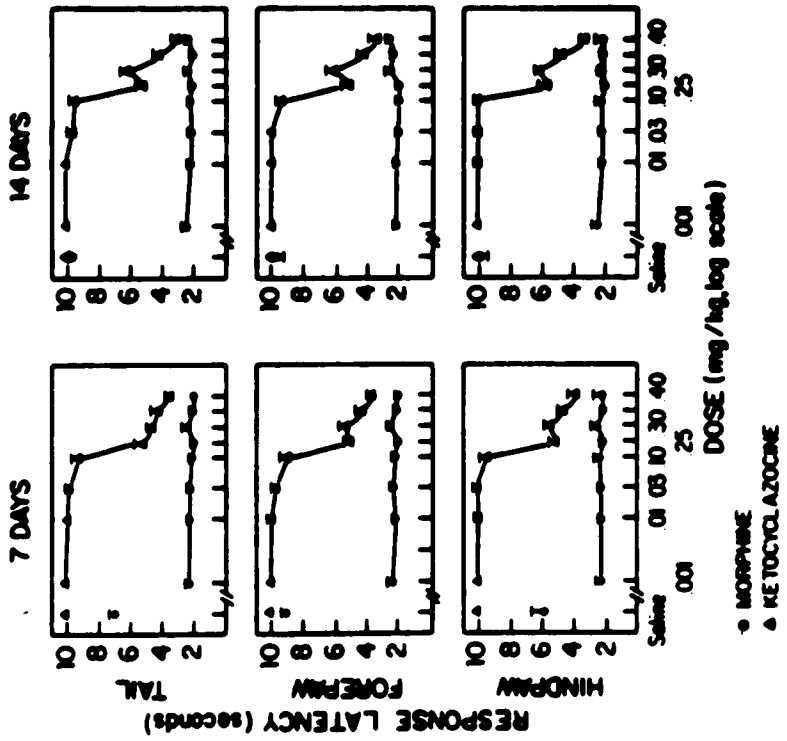

3 Days
5 Days
7 Days
10 Days
14 Days
Morphine
KC
FOREPAW
HINDPAW
TAIL
RESPONSE LATENCY (seconds)
10
8
6
4
2
Saline
Sal
1.0
3.0
10.0
DOSE mg/kg (log scale)

FIGURE 4. Naloxone reversal of morphine and KC-induced analgesia against a low intensity mechanical stimulus. Points represent mean response latencies (± SEM) to application of a 90^{o} angled blunt probe to the dorsal side of the forepaw, hindpaw and tail.

FIGURE 4


14 DAYS
7 DAYS
HINDPAW
FOREPAW
TAIL
RESPONSE LATENCY (seconds)
DOSE (mg/kg, log scale)
Saline
.001
.01
.03
.10
.30
1.0
2.5
4.0
MORPHINE
KETOCYCLAZOCINE

tested. In the hindpaw withdrawal tests, morphine was more effective than KC against higher intensity mechanical stimuli, while KC was more effective against a lower intensity stimulus. Neither drug was effective against thermally noxious stimulus applied to the hindpaw. Naloxone antagonized morphine-induced analgesia. Patterns of naloxone reversal of morphine- and KC-induced analgesia were similar for all body parts tested. I propose that the differences between morphine and KC reflect the differential abilities of these drugs to interact with mu and kappa opioid receptros. While neither drug is specific to a particular receptor type, the differential development, somatotopy, specificity to stimulus type and naloxone reversibility argue that they act through different receptors. The possibility that they interact with delta receptors has also been considered (Barr et al., 1983; Yaksh, 1984).

Further, morphine was found to be more effective against thermal and high intensity mechanical stimuli than KC. This suggests that both stimulus type and intensity may determine the pharmacological basis of opioid receptor-mediated analgesia. A model conceputalizing the neural circuitry involved in mu and kappa receptor-mediated analgesia in discussed in detail in Chapers 3 and 4.

The differential reversal of morphine- and KC-induced analgesia by naloxone provides further support for separate mediation of analgesia by mu and kappa opioid receptor systems. The absence of somatotopic differences in naloxone antagonism suggests both mu and kappa receptor involvement in nociceptive modulation of the forepaws, hindpaws and tail. The reversal of KC-induced sedation at lower

doses than KC-induced analgesia implies different receptor involvement for sedative and antinociceptive effects. One possibility is that KC may act through mu receptors to produce sedation, and spinal kappa sites to produce analgesia. A second possibility is that sedative effects are mediated by cortical kappa receptors, while analgesia is mediated by kappa receptors within the spinal cord. The action ofKC at cortical kappa sites may be more fully reversed by naloxone.

In conclusion, this study has demonstrated differences between morphine- and KC-induced analgesia that are dependent upon developmental factors, body topography, stimulus type and intensity. These data suggest that mu and kappa opioid receptor systems are differentially organized in the mediation of analgesia. Experiments presented in the following chapter attempt to define the physiological systems that subserve mu and kappa receptor mediated pain modulation in the developing rat.

CHAPTER THREE:

Role of Spinal 5-HT in mu and kappa Receptor-mediated Analgesia in the Developing Rat

INTRODUCTION

There is evidence that opiate drugs produce analgesia through distinct classes of opioid receptors at different neuroanatom,ical regions. While oppoing arguments exist (Porrecca et al., 1984), it appears that morphine acts through mu receptors at both the midbrain periaqueductal gray (PAG) and the spinal cord (Lewis et al., 1967; Wood et al., 1981; Yarsh et al., 1976), while keyocyclazocine (KC) (Wood et al., 1984) and U-50, 488H (vonVoighhtlander et al., 1984) act primarily through kappa receptors within the spinal cord. Several studies have addressed the possible neural mechanisms underlying mu- and kappa-opioid receptor-mediated analgesia. While there are conflicting data, pharmacologic and physiologic findings have suggested a role for serotonin (5-HT) in the expression of morphine-induced analgesia; although 5-HT involvement in KC-induced analgesia has also been proposed (vonVoigtlander et al., 1984a).

Intrathecal administration of the 5-HT receptor blocker methysergide attenuated morphine-induced analgesia (Yaksh 1978) and produced hyperalgesia against normal thermal noxious stimuli (Proudfit et al., 1981). Both morphine-induced (Tulanay et al., 1976) and central gray stimulation-induced analgesia (Carstens et al., 1983) were blocked by administration of the 5-HT synthesis inhibitor parachlorophenyl alanine (pCPA). Morphine-induced analgesia was reducwed by depletion of spinal 5-HT with the neurotoxin 5,6-dihydroxytryptamine (5,6-DHT) (Kuraishi et al., 1983; Proudfit et al., 1981). Lesions of the nucleus raphe magnus (NRM) dimished the analgesia effects of both systemic morphine (Azami et

al., 1978) and PAG stimulation (Behbehani et al., 1979). Intrathecally administered 5-HT (Wang, 1977) or 5-hydroxytryptophan (5-HTP) (Yaksh et al., 1979) increased nociceptive thresholds to thermal stimuli in both rat and cat. Additionally, morphine-induced analgesia was potentiated by subarachnoidal administration of the 5-HT re-uptake inhibitor citalopram (Larsen et al., 1982). Studies of Rivot and colleagues (1984) have demonstrated increased 5-HT biosynthesis in cord following subcutaneous administration of morphine. Spinal cord 5-HT turnover (Shiomi et al., 1978) and 5-hydroxyindoleacetic acid (5-HIAA) levels of (Messing et al., 1978; Weil-Fugazza et al., 1979) increased after systemic morphine administration. Further, elevated 5-HT release in the cord has been reported following microinjection of morphine to the PAG (Tyce et al., 1981; Yaksh et al., 1979). These results support the importance of 5-HT function in morphine-induced analgesia.

Analgesia produced by microinjection of morphine to the PAG (Wood et al., a1981), the paucity of direct PAG-spinal fibers and the involvement of 5-HT in morphine-induced analgesia further suggested a connection between the PAG and a descending bulbospinal 5-HT system. An excitatory PAG-NRM projection has been described (Fields et al., 1978). Spinal 5-HT tracts originating within the NRM and adjacent reticular nuclei descend along the dorsolateral funiculi (Basbaum et al., 1979; Bowker et al., 1981) and terminate in laminae I, II and V of the superficial dorsal horn (Ruda et al., 1980). PAG stimulation increased activity of neirons within the NRM (Morrow et al., 1984), depressed firing of nocisponsive neurons within the dorsal horn

(Dubuisson et al., 1980), and produced analgesia (Behbehani et al., 1979; Gray et al., 1983)

Several studies have demonstrated the post-natal, rostro-caudal maturation of this raphe-spina;l tract in several species, including the rat. Seroptonin-like immunoreactive fibers developed 3-5 days after birth in the cervico-thoracic cord, while 5-HT-like immunoreactivity in lumbo-sacral areas did not approach mature levels until 15 days post-partum (DiTirro et al., 1983; Ho, 1981). Serotonin-like immunoreactivity in laminae I and II of the cervical cord matured several days prior to lumbar regions (Gilbert et al., 1979; Martin, 1978). As the superficial dorsal horn is the site of primary nociceptive afferent input, as well as a probable locus of descending inhibition of nociceptive transmission (Basbaum et al., 1984), these data suggest progressive involovement of spinal 5-HT in the analgesia neuraxis in the developing animal.

Several groups have reported the selective engagement of the raphe-spinal system in analgesia against thermally noxious stimuli. NRM lesions (Abbot et al., 1982) or depletion of 5-HT in the cord with 5,6-DHT (Kuraishi et al., 1983) reduced morphine-induced analgesia against thermal pain. Intrathecal injection of 5-HT produced potent analgesia in hot-plate and tail-flick, but not tail-pinch tests of nociception (Kuraishi et al., 1985)

We have recently shown somatotopic and stimuli specific differences in the development of morphine and KC-induced analgesia in the preweanling rat (Giordano et al., 1984, 1985). These findings suggested maturation differences in the neural substrates subserving

mu- and kappaopioid recpetor-mediated analgesia. In this model, supraspinal mu receptors within the PAG interact with descending brainstem monoamine systems to produce analgesia; kappa receptors mediate analgesia segmentally within the cord, and may function independently of spinal monoaminergic involvement. The regional development of morphine-induced analgesia would depend upon the functional maturation of both mu- receptor and spinal monoamine systems, while the ontogeny of KC-induced analgesia would reflect development of kappa receptors within the cord.

The goal of the present experiments, therefore, was to provide direct eveidence for the role of spinal 5-HT in mu- and kappa-opioid receptor mediated analgesia dueing development. We depleted 5-HT in the spinal cord at 1 day of administered the 5-HT neurotoxin 5,7-DHT via intraspinal injection at 1 day of age and measured subsequent patterns of morphine and KC-induced analgesia against both thermal and mechanical stimuli in the developing rat.

MATERIALS AND METHODS

Subjects

Subjects were Long-Evans rat pups from rats mated in our colony. Pups were intraspinally injectwed 5,7-DHT within 24 hour of birth and returned to the dam. analgesia testing was performed at 7, 10 or 14 days of age. In earlier studies (Giordano et al., 1984, 1985) we ahve shown these ages to be critical points in the development of morphine- and KC-induced analgesia against thermal or mechanical stimuli. Pups were maintained at constant nest temperature

(33-35°C), and were permitted to have food, water and maternal contact and libitum prior to analgesia testing. The number of pups used was 6-8 per dose at each age, and each animal was used only once, and assignment to treatment and control groups was random.

Drugs

Drugs used were desmethylimipramine (DMI; Sigma), 5,7-dihydroxytryptamine (5,7-DHT; Sigma), morphine sulfate (generously supplied by Pennick Corporation) and ketocyclazocine (KC; generously supplied by Drs. Scott and Soria of Sterling-Winthrop Corporation). DMI, KC and morphine were dissolved in distilled water and were administered in a volume of 1 ml/100 g body weight. 5,7-DHT was dissolved in 0.2% ascorbate solution which also served as the vehicle for the intraspinal injections.

PROCEDURE

Neonatal Spinal 5-HT Depletion

A modification of the procedure described by Pappas et al. (1983) was used. Within 24 hours of birth pups were removed from the dam and administered 20 mg/kg of DMI i.p. to prevent uptake of 5,7-DHT by noradrenergic terminals. Forty (40) minutes after DMI administration, pups were lightly anesthetized with methoxyflurane (Metofane). Each animal was gently restrained and a small incision was made approximately 2-4 mm caudal to the intrascapular fat pds, allowing visualization of the vertebral column. A 30 ga. needle attached to a 10 ul syringe, oriented caudally was inserted to the

dorsal aspect of the vertebral column, just posterior to the fat pads. 0.5 ul of 8.0 ug 5,7-DHT dissolved in 0.2% ascorbate was delivered over a 70 second interval. Sham-treated animals received only DMI pretreatment and the ascorbate vehicle.

The incision was closed with cyanoacrylate cement, pups were allowed to recover fully from the anesthetic and were returned to the dam.

Analgesic Testing

Test procedures have been described previously (Giordano et al., 1984; 1985). Briefly, for mechanical testing, 5,7-DHT-treated and sham control pups aged 7 and 14 days (N=8 per dose x age) were administered either 0.0, 1.0, 3.0 or 10.0 mg/kg of morphine or KC i.p. All testing occurred 30 minutes post-injection. To assess mechanical analgesia, a blunt probe, fashioned from a dulled 23 ga. needle, bevelled to 90^{0}, 0.2 cm in diameter, was applied at 10 g of force to the dorsal side of the forepaw, hindpaw and tail, and withdrawal response latencies were recorded.

For thermal tests, treated and control pups aged 10 and 14 days (N=8 per dose x age) were administered either 0.0, 1.0, 3.0 or 10.0 mg/kg of morphine or KC i.p. Thirty (30) minutes post-injection the forepaw, hindpaw and tail were immersed in a bath of hot water (45^{0}C), and withdrawal response latencies were recorded. A cut-off of 10 seconds was used in both paradigms, after which the stimulus was discontinued. In these experiments, the less intense mechanical and themal stimuli were used. This allowed for more critical

assessment of 5,7-DHT treatment effects on morphine and KC-induced analgesia along several parameters: 1) extension of response baselines allowed more accurate comparison of analgesic effects between preweanling and adult rats; 2) increased response baselines permitted a more discriminable evaluation of possible motoric vs. analgesic effects of 5,7-DHT treatment; and 3) longer response baselines allowed for demonstration of even subtle changes in withdrawal latency produced by 5,7-DHT administration.

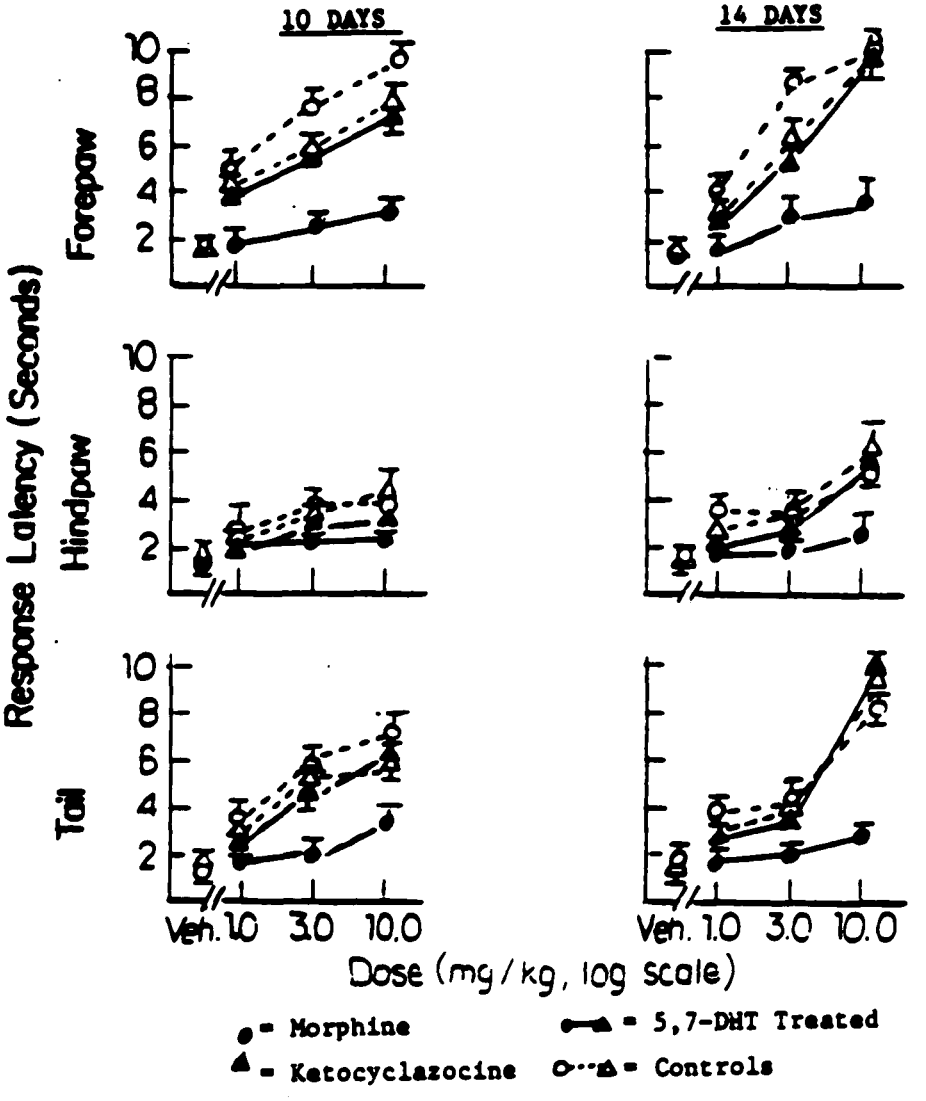
RESULTS

Thermal Tests

As n important focus of these experiments was the differential action of neonatal 5,7-DHT treatment on the analgesic effects of morphine and ketocyclazocine in thermal and mechanical tests of nociception, results are presented according to stimulus type. In all cases, data were statistically analyzed using 6-way factorial analysis of variance (ANOVA). Factors were: drug (morphine or ketocyclazocine), dose, treatment (5,7-DHT treated or control), age, bodypart (forepaws, hindpaws, tail) and stimulus type (thermal or mechanical). Data demonstrating effects of neonatal 5,7-DHT treatment on patterns of morphine- and KC-induced analgesia against thermal pain are shown in Figure 5. Consistent with previous findings (Giordano et al., 1984), in control animals, morphine effects preceded KC-induced analgesia in the forepaws, while KC-induced analgesia preceded morphine-induced effects in the hindpaws and tail by several days. Morphine first produced analgesia

FIGURE 5. Effects of neonatal intraspinal 5,7-DHT treatment on morphine- and KC-induced analgesia against a thermally noxious stimulus. Points represent mean withdrawal response latencies (±SEM) to immersion of the forepaw, hindpaw and tail into a bath of hot water (45°C).

FIGURE 5


10 DAYS
14 DAYS
Response Latency (Seconds)
Forepaw
Hindpaw
Tail
10
8
6
4
2
Veh.
1.0
3.0
10.0
Dose (mg/kg, log scale)
= Morphine
= Ketocyclazocine
= 5,7-DHT Treated
= Controls

in caudal body parts at 10 days of age, and these effects did not reach adult levels until day 14. In contrast, KC produced robust analgesia in all body parts tested at day 10.

Figure 5 demonstrates that neonatal administration of 5,7-DHT differentially affected morphine- an KC-induced analgesia (drug x dose x treatment interaction, $F[3,72] = 81.49$, $p.<.001$) and revealed a greater effects of 5,7-DHT treatment on morphine-induced than KC-induced analgesia (drug x stimulus x treatment x dose interaction; $F[3,72] = 58.75$, $p.<.001$).

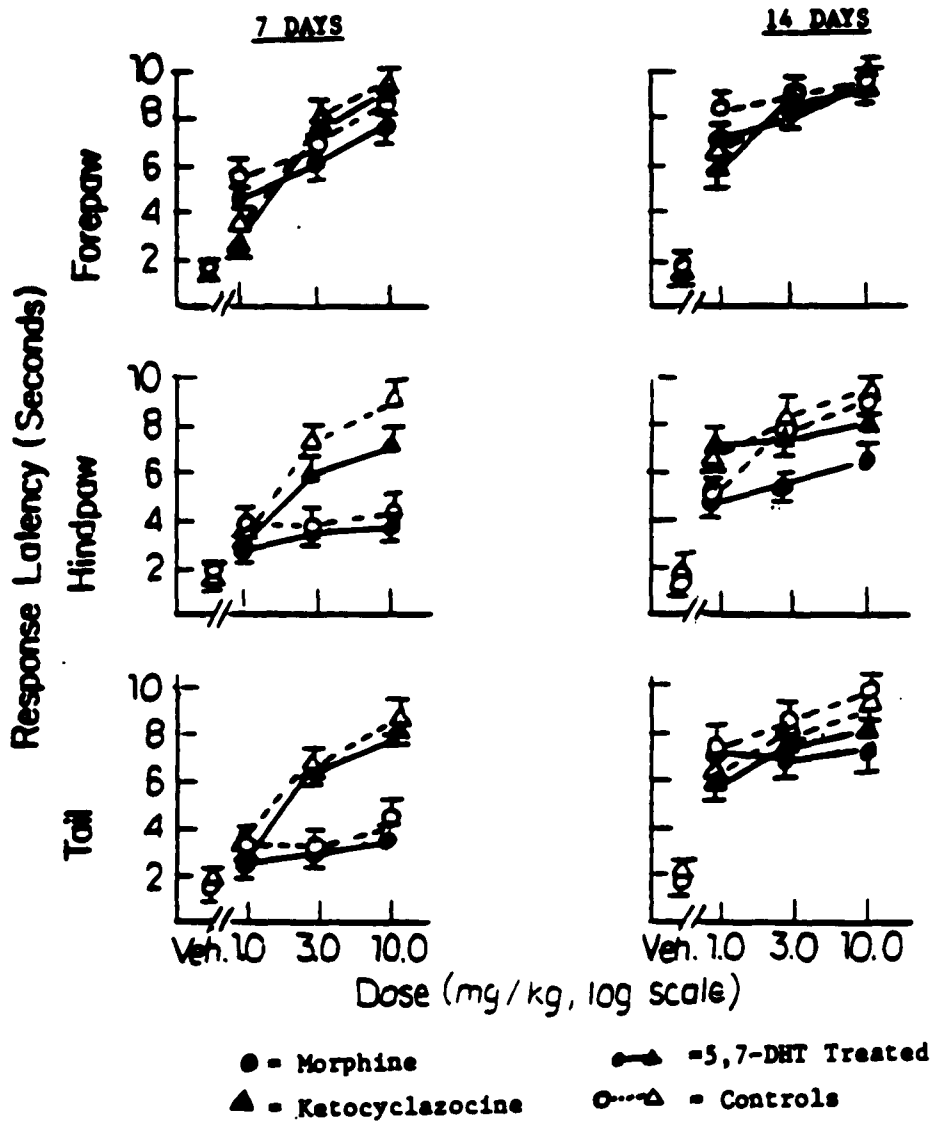
Further, these data demonstrated that treatment effects varied for body part tested; the effects of spinal 5-HT depletion were greatest in the forepaws and tail, while lesser effects were seen in the hindpaws (stimulus x drug x treatment x bodypart interaction; $F[2,48] = 34.91$, $p.<.001$) (refer to Figure 5). Serotonin depleted animals were hyperalgesic compared to controls as evidenced by decreased baseline withdrawal response latencies.

Mechanical Tests

Figure 6 presents the effects of neonatal 6,7-DHT treatment on morphine- and KC-induced analgesia against the mechanical stimulus. In control animals, morphine-induced analgesia in the hindpaws and tail first appeared at 7 days of age, and did not reach full potency until day 14. KC produced full analgesic effects in all body parts tested at 7 days of age (stimulus x drug x dose x age x treatment interaction, $F[6,144] = 11.17$; $p.<.001$; and by inspection of graphed mean data points). These findings are consistent with earlier

FIGURE 6. Effects of neonatal intraspinal 5,7-DHT treatment on morphine- and KC-induced analgesia against a mechanical noxious stimulus. Points represent mean withdrawal response latencies (± SEM) to application of a 90^{0} angled blunt probe to the dorsal side of the forepaw, hindpaw and tail.

FIGURE 6


7 DAYS
14 DAYS
Response Latency (Seconds)
Forepaw
Hindpaw
Tail
10
8
6
4
2
Veh.
1.0
3.0
10.0
Dose (mg/kg, log scale)
● = Morphine
▲ = Ketocyclazocine
=5,7-DHT Treated
= Controls

reports that have characterized the developmental patterns of morphine- and KC-induced analgesia against mechanically noxious stimuli (Giordano et al., 1985). As in thermal tests, 5,7-DHT treatment differentially altered patterns of morphine- and KC-induced analgesia (stimulus x drug x dose x treatment interaction, $F[3,72] = 58.75$; $p.<.001$). Again, spinal 5,7-DHT administration affected morphine-induced analgesia to a greater extent than KC-induced analgesia in all body parts tested (drug x dose x treatment x body part interaction, $F[6,144] = 15.14$; $p.<.001$).

Further, Figure 7 demonstrates that when cell means were collapsed over age, neonatal 5,7-DHT treatment more effectively reduced the analgesic potency of morphine in ther mal than in mechanical tests (stimulus x drug x treatment interaction, $F[3,72] = 342.97$; $p.<.001$).

DISCUSSION

Patterns of KC-induced analgesia in both thermal and mechanical tests were less significantly affected by spinal administration of 5,7-DHT. Figure 8 demonstrates that when cell means were collapsed over age groups, the effects of 5,7-DHT treatment on KC-analgesia were shown to be minimal in both thermal and mechanical paradigms.

Neonatal administration of 5,7-DHT differentially disrupted patterns of morphine-induced analgesia depending upon the nociceptive test. Against the thermal stimulus, 5,7-DHT treatment significantly reduced morphine-induced analgesia in all body parts at both ages tested. Against the mechanical stimulus, the analgesic potency of

FIGURE 7. Differential effects of neonatal intraspinal 5,7-DHT treatment on morphine-induced analgesia against thermal and mechanical stimuli. Points represent treatment or control cell means collapsed over age in order to demonstrate treatment x stimulus x drug interaction.

FIGURE 7

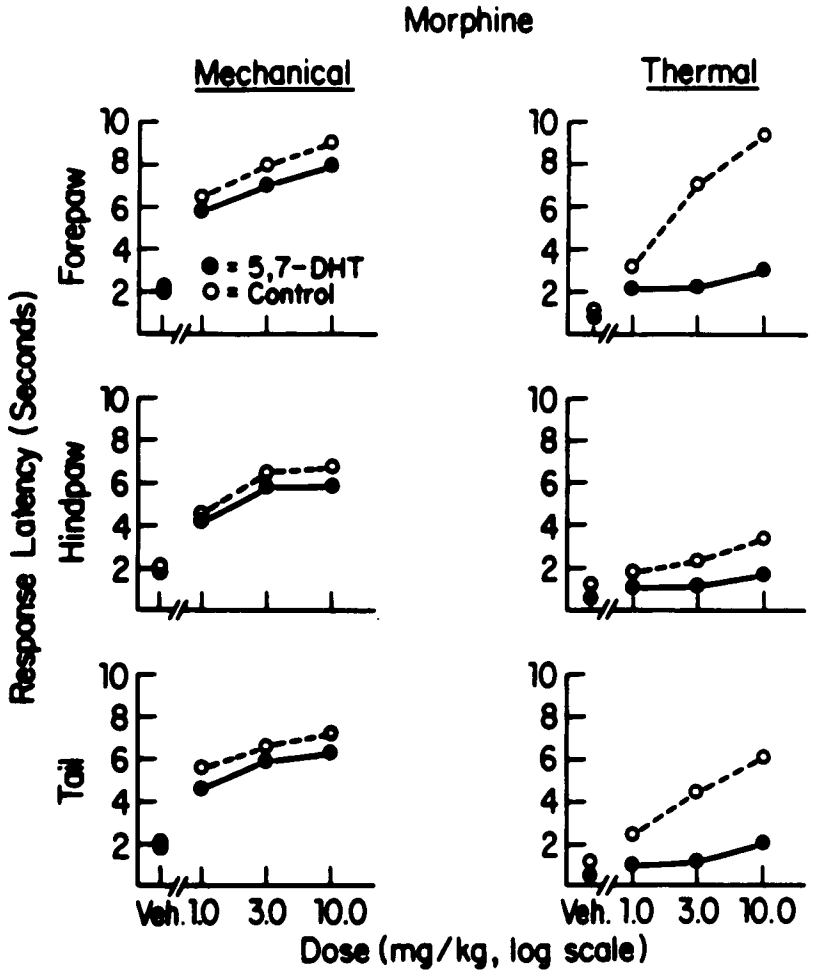
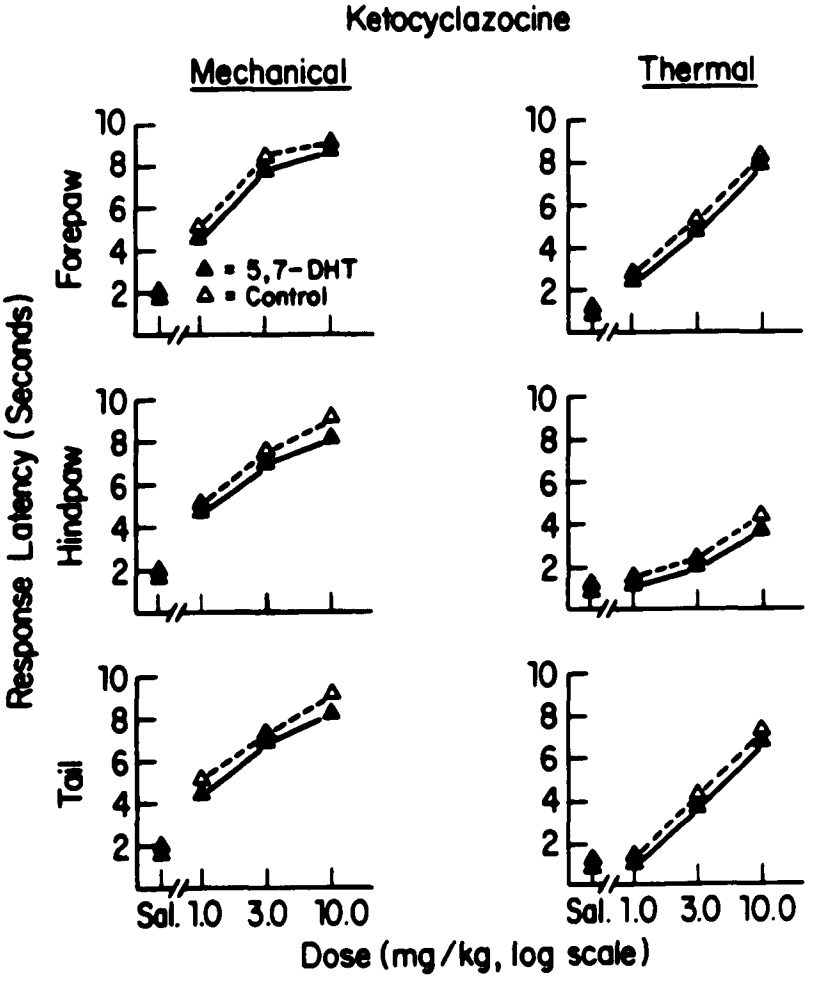

Morphine
Mechanical
Thermal
Forepaw
Hindpaw
Tail
● = 5,7-DHT
○ = Control
Response Latency (Seconds)
10
8
6
4
2
Veh. 1.0 3.0 10.0
Dose (mg/kg, log scale)

FIGURE 8. Differential effects of neonatal intraspinal 5,7-DHT treatment on KC-induced analgesia against thermal and mechanical stimuli. Points represent treatment or control cell group means collapsed over age in order to demonstrate treatment x stimulus x drug interaction.

FIGURE 8


Ketocyclazocine
Mechanical
Thermal
Forepaw
Hindpaw
Tail
▲ = 5,7-DHT
△ = Control
Response Latency (Seconds)
10
8
6
4
2
Sal. 1.0 3.0 10.0
Dose (mg/kg, log scale)

morphine were only slightly diminished. In animals that received the opiate vehicle, 5-HT depletion produced mild hyperalgesic effects in both thermal and mechanical tests.

These results demonstrate differential involvement of spinal 5-HT in morphine- and KC-induced analgesia. The reduction of the analgesic potency of morphine in thermal tests supports similar findings in the adult animal (Abbot et al., 1984; Kuraishi et al., 1984; Proudfit et al., 1982) that have suggested that a PAG-raphe-spinal 5-HT system may be selectively engaged to modulate thermal antinociception. Spinal 5,7-DHT administration less effectively attenuated morphine-induced analgesia against a mechanically noxious stimulus. However, morphine produced analgesia against mechanical input in control animals. Taken together, these results strongly suggest the possible involvement of some other brainstem mechanism in this effect. A recent study in our laboratory (Hughes et al., 1986) on the potency of intrathecal administration of the alpha-NE agonist clonidine against mechanical stimuli supports other investigations that have implicated a role for bulbospinal NE systems in mechanical analgesia (Kuraishi et al., 1985).

Patterns of KC-induced analgesia against both thermal and mechanical stimuli were less significantly disrupted by neonatal 5,7-DHT treatment. It is possible that KC produces analgesia independently of the spinal 5-HT system. This conclusion is in conflict with a report that the analgesic action of the novel kappa receptor agonist U-50, 488H was reduced by compounds that are 5-HT/2 receptor antagonists (e.g. - cyproheptadine, ketanserin) (Peroutka et

al., 1981). Concentration of 5-HT/2 receptors is high in cortex (Schotte et al., 1983) and low in the spinal cord (Monroe et al., 1983), while the analgesic locus of U-50, 488H action was at spinal kappa sites (Piercey et al., 1982). Thus, the reduction of U-50, 488H effects by 5-HT/2 receptor blockers may not reflect a direct 5-HT kappa receptor interaction within the analgesic neuraxis of the spinal cord, but may be due to antagonism of cortical processes involved in pain perception. Second, these studies only assessed the potency of U-50, 488H in thermal tests. As kappa receptor-mediated analgesia has been shown to be more effective against mechanical stimuli (Tyers 1980), nad the 5-HT system appears to more effectively mediate thermal analgesia (Kuraishi et al., 1983, 1985; Proudfit et al., 1982), it is possible to argue that 5-HT/2 receptor blockage may be reducing thermal analgesia through a neural circuit which is independent of the mechanisms subtending the kappa receptor system. Clearly, these details require further study.

In conclusion, the results of the present study suggest that the neural circuitry subserving mu- and kappa-opioid receptor-mediated analgesia may differ. This evidence supports the model of a sgemental spinal mechanism of kappa receptor-mediated analgesia and implies that multiple descending monoamine systems are responsible, in part, for patterns of mu-receptor-mediated analgesia in the preweanling rat.

CHAPTER FOUR

General Discussion

GENERAL DISCUSSION

Stimulus Specificity

Both morphine and KC are effective analgesics in the neonatal rat. In the developing animal, marked differences were apparent in the potencies of these agents against cutaneous thermal or mechanical pain. These results support the concept that morphine and KC act at distinct opioid receptor populations, previously defined as mu and kappa (Martin et al., 1976), and suggest a discriminable ability of these receptor systems to modulate different classes of nociceptive input. The analgesic potency of morphine against thermal stimuli was consistently greater than KC, particularly in the forepaws. Against mechanical stimuli, KC produced equipotent or greater analgesic effects than morphine at younger ages in caudal body parts.

These findings are consistent with existing data for adult animals that demonstrated tha mu receptor agonists were highly effective in tail-flick and hot-plate (i.e. - thermal) tests, while kappa agonists, such as KC and U-50, 488H, were considerably more active in mechanical or visceral chemcial tests (Abbott et al., 1982; Lahti et al., 1984; Piercey et al., 1982; Tyers, 1980). Several explanations may be proposed for these findings. One possibility is that parameters other than stimulus-type may determine the engagement of the mu or kappa opioid-receptor system. The efficacy of mu agonists in thermal and high intensity mechanical algesic paradigms may reflect that these stimuli may be a more complex sensory phenomenon than lower intensity stimuli. For example, although stimulus intensities were equilibrated according to withdrawal

response baselines, the sensory parameters induced by the application of distinct strengths of stimuli may involve different neural processes. A recent report by Price (1984) has shown that noxious heat involves a greater dermal area and may induce spatial and/or temporal summation in second-order nociceptive-specific afferents of the spinal cord. The potency of morphine against both thermal and high intensity mechanical stimuli supports this hypothesis.

Kappa receptor-mediated analgesia may not be effective against high intensity pain. Chemical evoked writhing and mechanical tests have generally been considered to represent weaker nociceptive stimuli (Blumberg et al., 1965; Taber, 1974). The effectiveness of kappa receptor agonists, in addition to the lower ED50 of morphine in these tests (Yaksh, 1984) supports the concept of kappa receptor modulation of less intense pain.

A recent study by Basbaum et al. (1986) may provide an anatomical basis for this hypothesis; Dynorphin was shown to be co-released with substance-P from primary afferents. Given the localization of kappa receptors within the superficial dorsal horn (Slater et al., 1984), and that dynorphin may be an endogenous ligand for the kappa receptor (Chavkin et al., 1982), it is possible that the kappa receptor may function pre-synaptically as an autoreceptor for dynorphin that is co-released from primary afferents. Conceptually, A-delta/C afferents might regulate their response output through kappa receptor mediated inhibition within a specific range of stimulus intensities. As stimulus intensity increases, kappa receptor-mediated inhibition would be less effective.

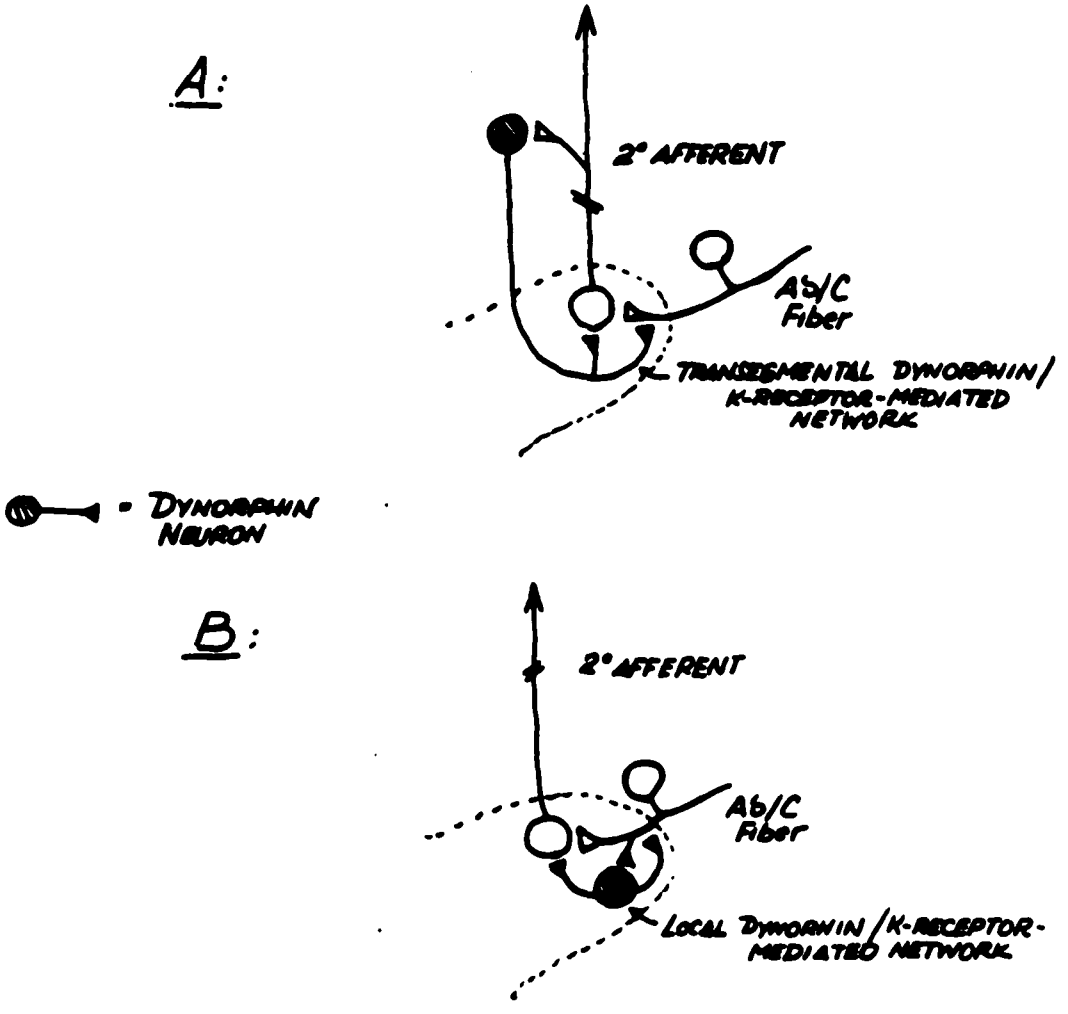
Another possibility is that low frequency A-delta/C fiber activity might evoke a local or transegmental dynorphinergic circuit within the cord to engage kappa receptor-mediated modulation of low intensity noxious input. Similarly, this type of inhibition may be most effective against a punctate or weak noxious noxious stimulus taht does not involve several dermatomes and/or invoke a diffuse summating response in second order afferents (see Figure 9).

A second hypothesis is that nociceptive pathways are anatomically and functionally segregated according to stimulus type. Noxious heat may activate a thermally-specific dorsal horn pathway to engage primarily a mu opioid receptor-mediated analgesic network. Mechanical pain may activate kappa receptor-mediated mechanisms within the spinal cord. It may also be that kappa receptors are associated with mechano-specific afferents to a greater extent than with thermal nocisponsive units. These concepts are speculative interpretations of the data. Further investigation (i.e. - specific ultrastructural localization of receptor types on specific primary and/or secondary afferent networks) is required to clarify the roles of these opioid receptor systems in the modulation of specific types of pain.

The pharmacological profiles and analgesic potencies of mu and kappa opiates cannot entirely "define" that drug actions are mediated through separate receptor types. A recurrent caveat is that opiate agonists have different affinities at several receptor types. However, one strategy to strengthen the assumption that the action of an opiate agonist is mediated by a particular receptor system is to

FIGURE 9. Possible dynorphinergic/kappa opioid receptor-mediated analgesic circuitry within the dorsal horn of the spinal cord; Figure A represents the activation of a transegmental dynorphin network by A-delta/C fiber and nocisponsive second order afferent input. Figure B represents a local dynorphin circuit in which A-delta/C fiber activity evokes a segmental dynorphinergic/kappa receptor-mediated analgesia. In both arrangements, dynorphin acts at kappa sites located on A-delta/C fiber terminals and/or perikarya of nocisponsive second order afferents.

FIGURE 9


A:
2° AFFERENT
Aδ/C Fiber
TRANSSEGMENTAL DYNORPHIN/ K-RECEPTOR-MEDIATED NETWORK
= DYNORPHIN NEURON
B:
2° AFFERENT
Aδ/C Fiber
LOCAL DYNORPHIN/K-RECEPTOR-MEDIATED NETWORK

examine the differential reversal of those actions by the antagonist naloxone.

The affinity of naloxone for the mu receptor is at least one order of magnitude greater than for the kappa site (Martin, 1979), and pA2 values differ for morphine and KC (Tallarida et al., 1978). In the developing rat, high doses of intraperitoneally administered naloxone were required to reverse the analgesic effects of KC in all body parts tested. At he highest range, this antagonism was dose-dependent. In contrast, complete reversal of morphine-induced analgesia wasseen at all doses of naloxone. The dose-response analysis of naloxone antagonism provided additional support that the specific analgesic effects produced by morphine and KC were mediated by distinct mu and kappa receptor populations in the preweanling rat.

Developmental Differences

In both thermal and mechanical tests, differences existed in the developmental patterns of morphine and KC-induced analgesia. Briefly, morphine-induced analgesia preceded KC-induced analgesia in the forepaws against both thermal and mechanical nociception, while KC effects occurred several days prior to morphine-induced analgesia in the hindpaws and tail in these tests. KC produced potent analgesia in all body parts tested between 7 and 10 days of age, in mechanical and thermal tests, respectively. Morphine-induced analgesia was first seen in the forepaws at 3-5 days of age, depending upon the test, however, morphine effects in hindpaws and tail were minimal until 14 days postnatally.

These developmental patterns strongly suggest the differential maturation of distinct mu and kappa opioid receptor systems in the preweanling rat. The patterns of morphine- and KC-induced analgesia may reflect the development of mu and kappa receptors at different ages throughout the spinal cord. In this scheme, mu receptors might mature progressively in a caudal direction within the cord. Thus, mu receptor-mediated analgesia would be seen in rostral areas (i.e. - forepaws) several days prior to caudal regions (i.e. - hindpaws, tail). The kappa receptor system might not assume this rostro-caudal pattern of development, and may mature somewhat uniformly throughout the cord within the first postnatal week. Therefore, the onset of kappa receptor-mediated analgesia in rostral regions might occur later than mu receptor-mediated effects, while kappa receptor-mediated analgesia would precede mu receptor-mediated analgesia in caudal structures (i.e. - hindpaws, tail). Although this model is attractive, several lines of evidence implicate a more complex system. First, spinal transection attenuated morphine-induced, but not KC-induced analgesia (Wood et al., 1981). Second, morphine administered centrally to the PAG produced potent analgesia, while KC was not analgesic when administered to that site (Wood et al., 1981). These findings argue that morphine-induced analgesia involves some supraspinal component, while KC-induced analgesia does not. Third, the paucity of mu receptors in the lumbo-sacral spinal cord (Gourderes et al., 1982) suggests that some factor other than spinal mu receptors may be at least partially

responsible for the late development of morphine-induced analgesia in caudal body parts.

Considering these data, I have proposed a model in which morphine acts primarily through supraspinal mu sites within the midbrain PAG. Expression of morphine-induced analgesia appears to be dependent in part, upon the activation of one or more brainstem monoaminergic systems which descend in the spinal cord. KC may not act through this system, but rather activates populations of kappa sites within the cord to produce segmental analgesia. Based upon this model, I hypothesize that the patterns of analgesia produced by morphine and KC in the developing animal reflect the maturation of the neural mechanisms which subserve mu and kappa receptor mediation of pain.

The development of morphine-induced analgesia would be dependent upon the maturation of both mu-opioid receptors and descending bulbospinal systems. The ontogeny of KC-induced analgesia, if independent of descending brainstem systems, would require only the regional maturation of kappa sites within the spinal cord.

However, questions arise as to what bulbospinal systems are involved with mu receptor-mediated analgesia, and whether the kappa receptor system is at all reliant upon these descending mechanisms for the expression of KC-induced analgesia in the developing rat. To address these issues, it was decided to examine the effects of neonatal 5,7-DHT treatment on morphine- and KC-induced analgesia in the neonatal animal. If the neural mechanisms involved in mu and kappa receptor-mediated analgesia were distinct, neonatal 5,7-DHT

treatment should differentially affect developmental patterns of morphine- and KC-induced analgesia.

Role of 5-HT in Mu and Kappa Receptor Mediated Analgesia

The results presented in this thesis build upon the model that implicates a role for brainstem monoamines in opiate analgesia (see Chapter 1 for review). Neonatal administration of 5,7-DHT decreased morphine-induced analgesia against thermal pain to a greater degree than mechanical pain. Yet morphine produced analgesic effects against mechanical stimuli, and the developmental pattern of morphine-induced analgesia differed in thermal and mechanical tests. These findings indicate a differential function of spinal 5-HT in morphine-induced analgesia. It may be that morphine-induced mechanical analgesia is subserved primarily by a non-5-HT brainstem pathway. Noradrenergic involvement in mechanical analgesia has been shown in preweanling (Hughes et al., 1986) and adult animals (Kuraishi et al., 1983, 1985). Neuroanatomical evidence further argues for the involvement of both 5-HT and NE bulbospinal systems in opiate analgesia; 5-HT cell bodies are located within the medullary NRM. The nuclei of origin of NE neurons are also in the rostro-ventral medulla and caudal pons, specifically the nuclei reticularis gigantocellularis (NRGC) and paragigantocellularis (NRpG) (Dahlstrom & Fuxe, 1965). Both systems receive input from the PAG (Abols et al., 1981), and project to the dorsal horn of the spinal cord via the dorsolateral funiculi (Basbaum et al., 1984).

It is possible to envision segregated thermal and mechanical afferent projections to the PAG that selectively activate a particular PAG-brainstem system. These multiple PAG-bulbospinal mechanisms might subtend mu receptor-mediated analgesia specific to the type of noxious input. Thus, noxious heat may activate a PAG-NRM-spinal circuit, while mechanically noxious stimuli might engage a PAG-NRGC/pG-spinal neuraxis. These factors may provide a possible explanation for the somatotopic and developmental differences in morphine-induced analgesia against thermal and mechancial pain (see Figure 10).

The ontogeny of the descending 5-HT and NE tracts are not identical (DiTirro et al., 1983; Ho, 1981; Jonsson et al., 1982). Therefore, the differnt time-courses of development seen in patterns of morphine-induced analgesia against thermal and mechanical noxious stimuli may be due, in part, to dissimiliar innervation of spinal targets by 5-HT and NE systems during maturation. Once mature, these systems may function separately or in tandem in mu receptor mediated analgesia against various types of pain (see Figure 11).

Inspection of the data revealed that the effects of neonatal 5,7-DHT treatment were greater for morphine- than KC-induced analgesia (refer to Figures 7, 8); KC-induced analgesia was less significantly affected by neonatal administration of 5,7-DHT; thus suggesting that spinal 5-HT function may be less critical for the expression of kappa receptor-mediated analgesia. The possibility that a NE pathway interacts with the kappa receptor system to produce analgesia has been addressed elsewhere (vonVoigtlander et al., 1983).

FIGURE 10. Schematic representation of segregated thermal and mechanical nociceptive pathways that activate distinct mu receptor populations within the PAG to engage multiple bulbospinal circuits. In this model, thermal input activates a PAG-raphe-spinal (5-HT) mechanism, while mechanical input activates a PAG-NRGC/pG-spinal (NE) mechanism.

FIGURE 10

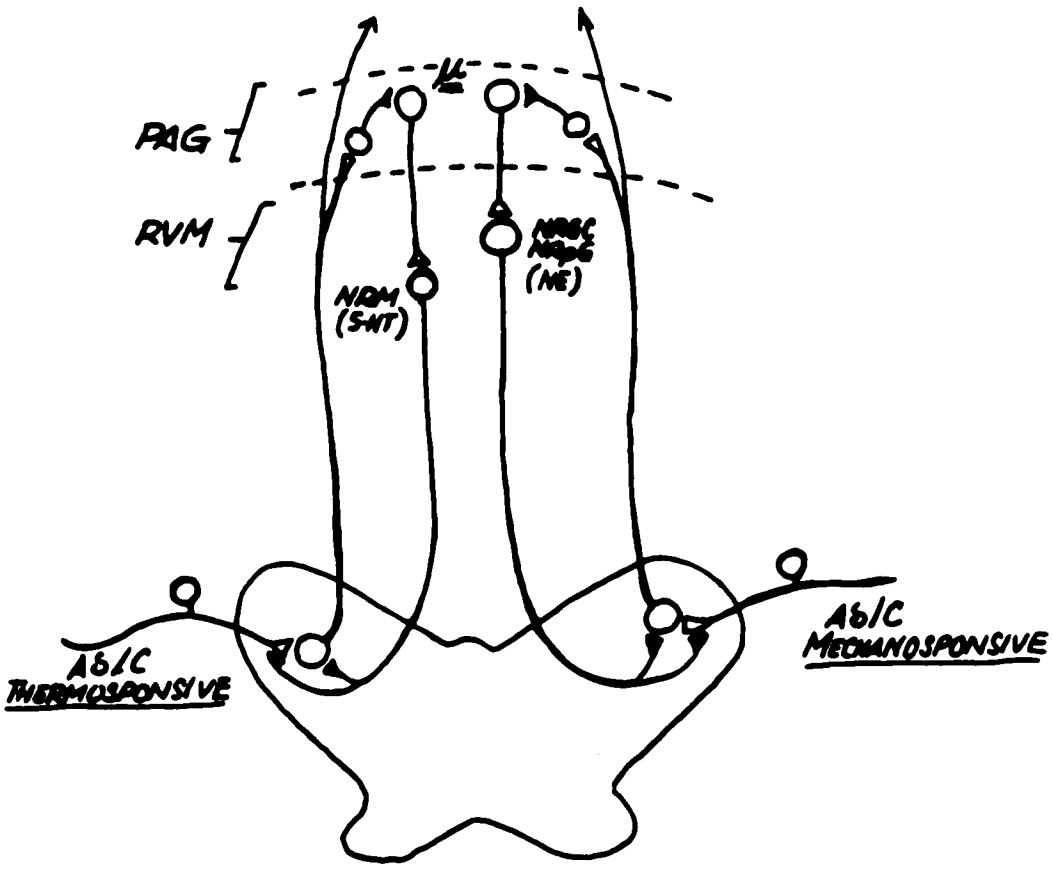
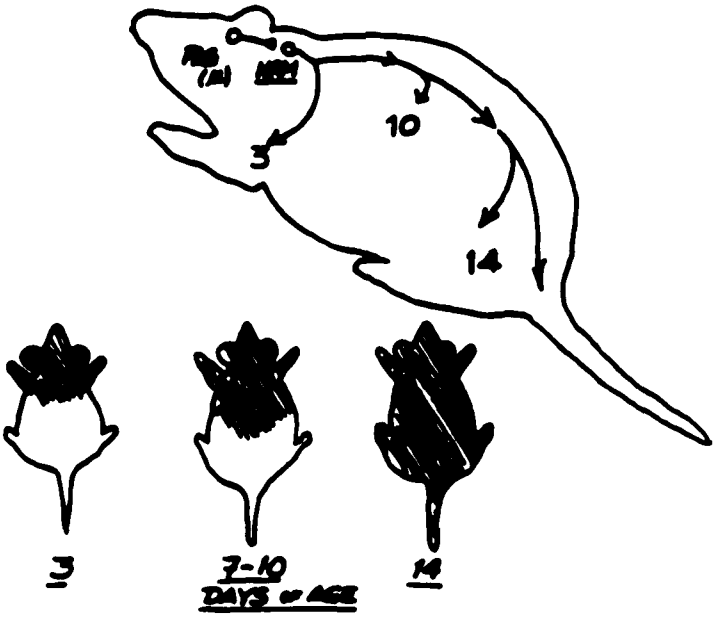


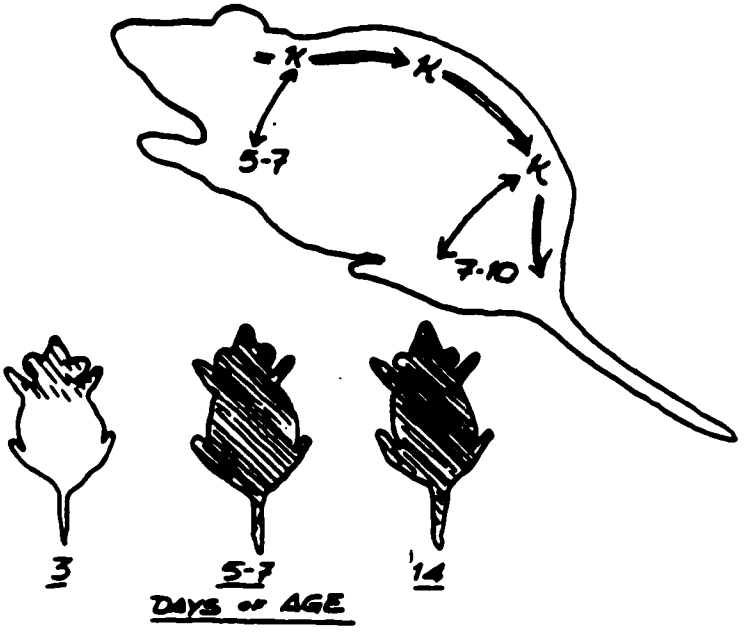
FIGURE 11. Diagrammatic representation of differential time-course of morphine- and KC-induced analgesia in the developing rat. These developmental differences in patterns of analgesia produced by morphine and KC may reflect distinct maturational processes of the neural mechanisms that subserve mu and kappa opioid receptor-mediated analgesia.

FIGURE 11

A:



B:



Prazocin, an alpha-NE receptor antagonist failed to reduce the analgesic potency of the kappa agonist U-50, 488H (vonVoigtlander et al., 1983, 1984), suggesting that NE mechanisms are not involved with kappa receptor-mediated analgesia.

Taken together, these data support the model of a segmental spinal mechanism of kappa receptor-mediated pain modulation that functions somewhat independently of bulbospinal monoaminergic systems. In agreement with earlier studies (Basbaum et al., 1984; Fields et al., 1978), I propose that mu receptor-mediated analgesia involves, in part, an interaction between supraspinal mu opioid and brainstem monoaminergic systems. The roles of the descending monoamine systems functional in mu receptor-mediated analgesia appear to depend upon the type of noxious input. Brainstem 5-HT mechanisms are more analgesic against thermal pain; brainstem NE mechanisms are more effective against mechanical stimuli.

Further, the potency of mu receptor-mediated analgesia against different types of pain might reflect the regional involvement of 5-HT and/or NE circuitry. The 5-HT or NE innervation of specific body regions may be responsible for topographic differences in the analgesic properties of morphine in thermal and mechanical tests. Additionally, the distribution of mu and kappa opioid receptor systems in the nociceptive neuraxis may also contribute to distinct patterns of stimulus-specific analgesia. Thus, certain body regions may be considered to be subserved by one receptor system rather than another. The different analgesic patterns produced by morphine andKC

strongly support the concept of separate neural circuitry for mu and kappa receptor systems.

Such differences are clearest in the neonatal animal, where the actions of mu and kappa opiates produce analgesic profiles that reflect the heterogeneous development of these systems. As these systems mature, the neural mechanisms contributing to the unique properties of regional analgesia and stimulus-specificity become more difficult to clarify. This may indicate that although these endogenous analgesic systems are pharmacologically and physiologically distinct, they function synergistically to modulate pain in the adult animal.

The present studies have demonstrated differences in patterns of development, stimulus-specificity, naloxone reversibility and 5-HT involvement in morphine- and KC-induced analgesia in the preweanling rat. These results support the hypothesis that the analgesic effects of morphine and KC are mediated by the mu and kappa receptor systems, respectively, and are consistent with existing pharmacologic (Piercey et al., 1982; Wood et al., 1981; Yaksh, 1978) and anatomical data (Goodman et al., 1982; Slater et al., 1983) that suggested a spinal analgesic locus of kappa agonist action and a supraspinal/spinal effect of mu receptor agonists. While the present study addressed the function of mu and kappa receptor systems in analgesia, it should be noted that the delta opioid receptor has recently been shown to mediate analgesia at the spinal level as well (Schmauss et al., 1983; Tung et al., 1982; Yaksh, 1984). Additionally, the involvement of several otehr neurochemical systems, (e.g. - neurotensin) in

analgesia have been demonstrated (see Basbaum et al., 1984 for a review). These findings indicate further the complex nature of opioid receptor-mediated analgesia. The multiple sites at which various endogenous opioids, opiate drugs and monoamines act to produce antinociception illustrate that pain modulation occurs through several mechanisms at different levels within the central nervous system.

The utility of such studies is to further "unravel" the endogenous analgesic networks so as to develop more specific and selective clinical approaches to pain therapeutics.

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