Title

The contribution of the anti-opioid peptide cholecystokinin to morphine analgesia and tolerance

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The Contribution of the Anti-Opioid Peptide Cholecystokinin to Morphine Analgesia and Tolerance

by

Jennifer M. Mitchell

DISSERTATION

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Acknowledgments

There are several people without whose help this thesis would not have been possible. The first and foremost is my thesis advisor Howard Fields, who gave me the freedom to pursue the topics and conduct the experiments that interested me, even when my ideas differed entirely from his own. My thesis chair, Allan Basbaum, has been a boundless source of knowledge and humor and has been instrumental in shaping this thesis. I am also grateful for the help and encouragement that I received from the members of my thesis committee: Mary Dallman, Larry Tecott, and Linda Watkins. Had it not been for their enthusiasm, the ideas presented herein may have never come to fruition.

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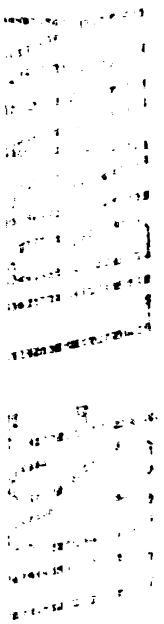
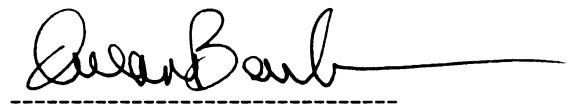


The Contribution of the Anti-Opioid Peptide Cholecystokinin to Morphine Analgesia and Tolerance

by Jennifer Mitchell



Allan Basbaum

Chair, Thesis Committee

Abstract

Cholecystokinin (CCK) is an anti-opioid peptide that antagonizes the analgesic effects of morphine and impedes the acquisition of morphine tolerance. This thesis extends upon previous research by using a combination of behavioral, pharmacological, and immunohistochemical techniques to investigate the role of CCK in morphine analgesia and tolerance and to ascertain the contribution of CCK to conditioned learning.

We demonstrate that following the administration of systemic morphine, the microinjection of CCK into the rostral ventromedial medulla (RVM) attenuates morphine analgesia in awake, freely moving rats. Additionally, we show that CCK, acting at the CCK-B receptor, is required for the expression of associative (conditioned), but not non-associative (non-conditioned), morphine tolerance.

Furthermore, we report that the lateral and basolateral nuclei of the amygdala are critical targets of this CCK effect. Lastly, we show that, following exposure to a context which has been repeatedly paired with morphine administration, Fos immunoreactivity is increased in the lateral and basolateral amygdala as well as in area CA1 of the hippocampus.

Taken together, the data presented in this thesis support the hypothesis that the amygdala and hippocampus are critically involved in the expression of associative morphine tolerance and suggest that these two nuclei may be acting together to create an association between a specific environment and the rewarding effects of morphine administration. Our data also suggest that CCK not only attenuates the effects of acute morphine administration but also participates in the expression of a learned compensatory response which serves to antagonize the effects of repeated morphine administration. Given the current results, we hypothesize that morphine overdose may in part be due to a failure of associative tolerance when an animal is administered morphine in a context other than that in which the animal has learned to anticipate morphine administration.

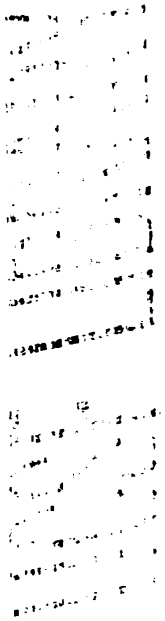
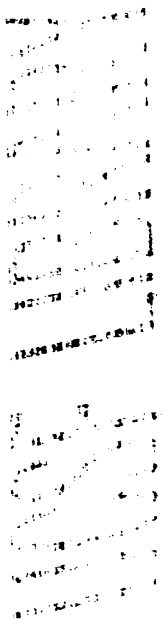


Table of Contents

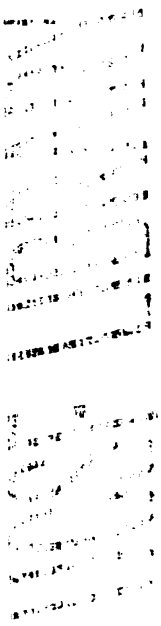
List of Figures



General Introduction

Directive

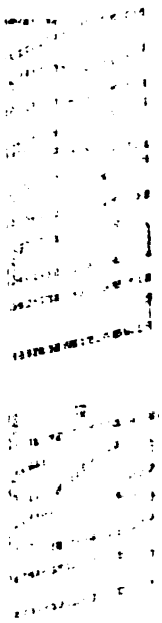
The purpose of this thesis is to investigate the contribution of the rostral ventromedial medulla (RVM) to analgesia in stressed versus unstressed animals and, additionally, to better elucidate the role of the anti-opioid peptide cholecystokinin (CCK) in morphine-induced analgesia and tolerance. The goal of this thesis is to more fully understand the processes by which analgesia is modulated under different experimental and behavioral conditions.



Objectives

The preliminary objective of this thesis was to investigate the contribution of stress to pain modulation at the level of the brainstem. The most extensively studied circuit involved in pain modulation includes the thalamus, amygdala, periaqueductal gray, RVM, and spinal cord (Fields, 1987; Figure 1). Ascending information is carried from the dorsal horn of the spinal cord through the spinal thalamic tract (STT) to the RVM and thalamus. Descending information travels from the cortex through the amygdala and periaqueductal gray (PAG) before reaching the RVM. From there information travels through the dorsolateral funiculus (DLF) to the spinal cord.

The RVM is a critical component of this circuit. Both electrical stimulation of the RVM and the microinjection of morphine into the RVM produce analgesia (Zorman, Hentall, Adams, & Fields, 1981; Jensen & Yaksh, 1986a; Jensen & Yaksh, 1986b). Additionally, inactivating or lesioning the RVM blocks the analgesia associated with PAG stimulation or morphine microinjection (Behbehani & Fields, 1979; Gebhart, Sandkuhler, Thalhammer, & Zimmerman, 1983; Young, Watkins, & Mayer, 1984), and the stress-induced potentiation of morphine analgesia (Kelly & Franklin, 1984; Fleetwood & Holtzman, 1989). RVM stimulation also inhibits nociceptive neurons in layers I, II, and V of the spinal cord dorsal horn (Fields, Basbaum, Clanton, & Anderson, 1977; Willis, 1982). Furthermore, the RVM is the primary source of dorsal horn serotonin (Dahlstrom & Fuxe, 1964), a neurotransmitter known to be important for modulation of nociceptive transmission (Jordan, Kenshalo, Martin, Haber, & Willis, 1978; Yaksh & Wilson, 1979; Berge, Hole, & Ogren, 1983; Roberts, 1984). Although spinal serotonin clearly contributes to nociceptive responsiveness in stressed animals, there is evidence that it does not contribute to analgesia in unstressed animals (Kennett & Joseph, 1981; Milne & Gamble, 1990).



Taken together, the data suggest that the RVM is not only modulating the effects of endogenous and exogenous opioids but may also be contributing to stress-induced changes in analgesia. To determine whether the modulatory effects of the RVM are dependent on behavioral state, the RVM was reversibly inactivated with either muscimol or lidocaine in stressed and unstressed animals and the

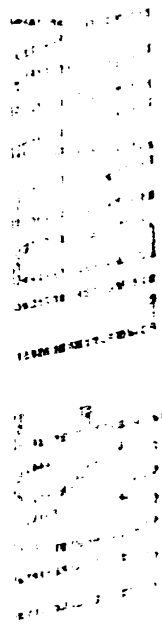
effects of morphine administration were assessed in these two groups of animals (Chapter 3).

The second aim of this thesis was to ascertain the effects of the neuropeptide cholecystokinin (CCK) on morphine analgesia. The role of CCK as an anti-analgesic peptide is well established. Systemically administered CCK attenuates morphine-induced antinociception, as do microinjections of CCK into either the PAG or spinal cord (Faris, Komisaruk, Watkins, & Mayer, 1983; Li & Han, 1989). CCK also modulates stress and anxiety. The i.v. administration of CCK to humans produces panic and this effect can be blocked by the administration of a CCK_B receptor antagonist (Bradwejn, Koszycki, Shriqui, & Meterissian, 1990; Abelson, & Nesse, 1990; De Montigny, 1989; Bradwejn, Koszycki, Couetoux du Tertre, Megen, Boer, Westenberg, & Annable, 1994; Lines, Challendor, & Traub, 1995). Given the presence of both CCK and its receptors in the RVM (Honda, Wada, Battey, & Wank, 1993; Skinner, Basbaum, & Fields, 1997) and the data indicating that the RVM is an important site for the modulation of stress-induced analgesia (Kelly et al., 1984; Maier, Grahn, Kalman, Sutton, Wiertelak, & Watkins, 1993; Watkins, Wiertelak, McGorry, Martinez, Schwartz, Sisk, & Maier, 1998), it appears that CCK may modulate nociceptive transmission at the level of the RVM. This thesis extends previous research by studying the effects of CCK microinjections into the RVM following systemic morphine administration (Chapter 3).

The third aim of this thesis was to determine the effects of the neuropeptide cholecystokinin (CCK) on the expression of two different types of morphine tolerance: associative (conditioned) and non-associative (non-conditioned). Because most investigators fail to distinguish between these two types of morphine tolerance when designing an experiment, the current literature surrounding the effects of CCK on morphine tolerance is rather convoluted. For instance, the dose of morphine, amount of time between morphine administrations, route of administration, and presence of salient environmental cues have all been shown to determine the type of morphine tolerance that develops (Tiffany & Maude-Griffin, 1988; Dafters & Odber, 1989; Grisel, Wiertelak, Watkins, & Maier, 1994). Several groups have found that the co-administration of a CCK antagonist with morphine can delay or temporarily prevent the acquisition of morphine tolerance (Tang, Chou, Iadarola, Yang, & Costa, 1984; Dourish, Hawley, & Iversen, 1988; Dourish, O'Neill, Coughlan, Kitchener, Hawley, & Iversen, 1990; Xu, Wiesenfeld-Hallin, Hughes, Horwell, & Hokfelt, 1992). However, since these groups tested the acquisition of morphine tolerance over short time periods (1-6 days), used short intervals between morphine administrations (2-8 hours), administered progressively higher doses of morphine (3-48 mg/kg), used different CCK antagonists, and different routes of drug administration, it is impossible to know to which type of morphine tolerance their results pertain. Careful review of the literature suggests that the results of these studies are best explained by changes in learning and conditioning resulting from specific factors in experimental design. Therefore, one objective of

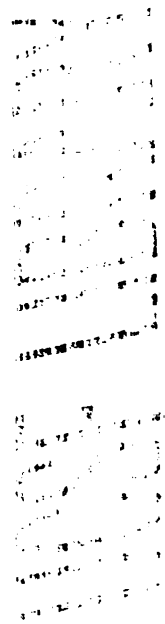
this thesis was to study the differential effects of CCK and conditioning on associative and non-associative morphine tolerance. To this end, two selective CCK antagonists were systemically administered following the acquisition of either associative (conditioned) or non-associative (non-conditioned) morphine tolerance (Chapter 4).

The final objective of this thesis was to examine the circuitry involved in associative versus non-associative morphine tolerance. Since Pavlovian conditioning has been shown to contribute to associative, but not non-associative, tolerance (Baker & Tiffany, 1985; McLaughlin, Dewey, & Fanselow, 1991), it was possible to hypothesize that brain regions involved in learning the emotional salience of reinforcers might contribute differently to these two forms of tolerance. The nucleus accumbens, ventral tegmental area, hippocampus, and amygdala are all involved in the modulation of reward and expectancy and are necessary for stimulus-response pairings (Spiegler & Mishkin, 1981; LeDoux, 1986; Wise, 1987; Wise & Rompre, 1989; Figure 2). Therefore, it seemed likely that differences between associative and non-associative morphine tolerance would be found within one or more of these nuclei. To this end, Fos immunoreactivity was used as a marker of neuronal activity following the acquisition of either associative or non-associative morphine tolerance and subsequent exposure to either a drug-paired or neutral environment (Chapter 5).



Summary

This thesis expands upon the current literature regarding nociceptive modulation by investigating the role of the rostral ventromedial medulla (RVM) in stressed and unstressed animals. Additionally, this thesis attempts to determine the effects of CCK on opioid-induced analgesia in the RVM and on associative and non-associative morphine tolerance. Lastly, this thesis investigates differences in brain regions involved in associative and non-associative morphine tolerance.



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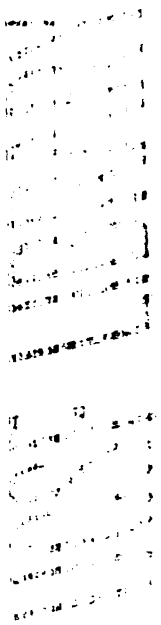


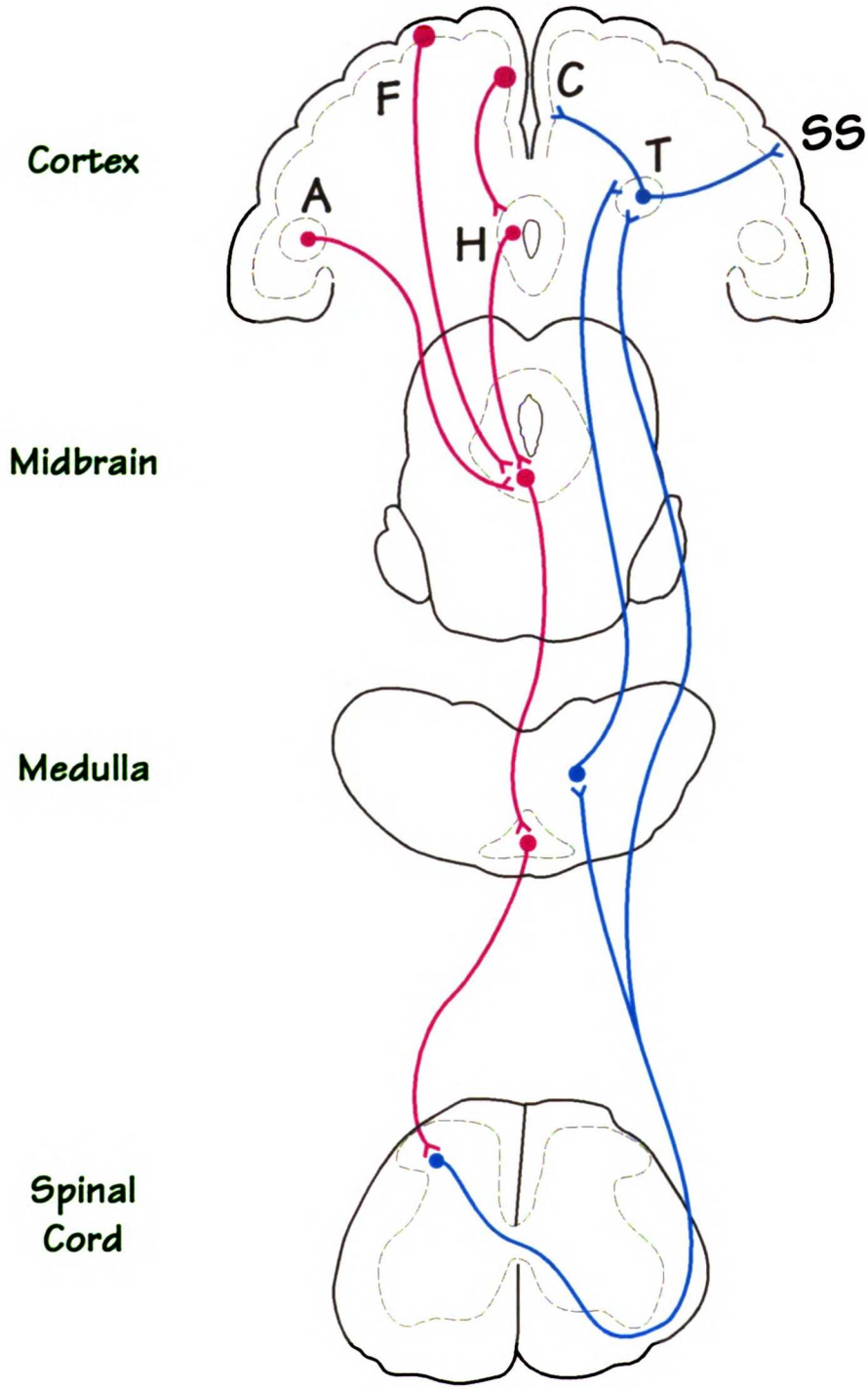


Figure 1: The ascending and descending pain modulatory pathway. Ascending information travels from the dorsal horn of the spinal cord to the brainstem and thalamus while descending information travels from the amygdala through the midbrain and brainstem to the dorsal horn of the spinal cord. A= amygdala, F= frontal cortex, C= cingulate cortex, SS= somatosensory cortex, H= hypothalamus, and T= thalamus.

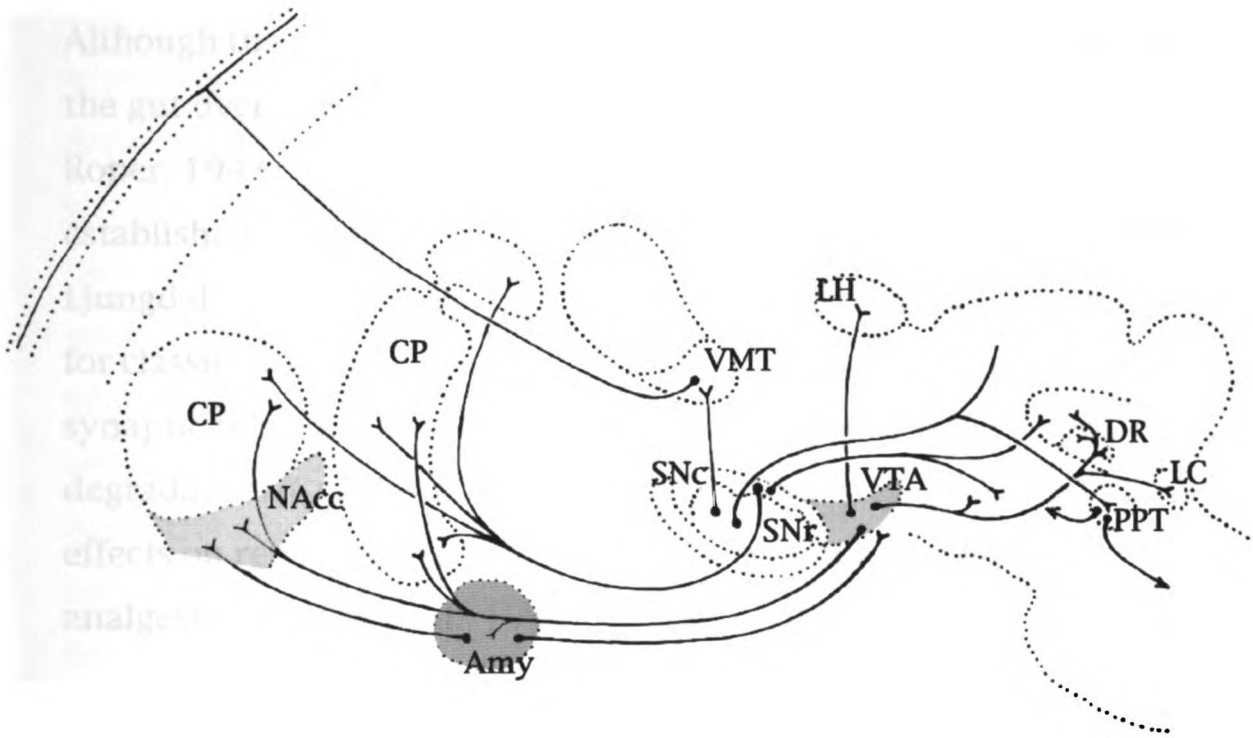
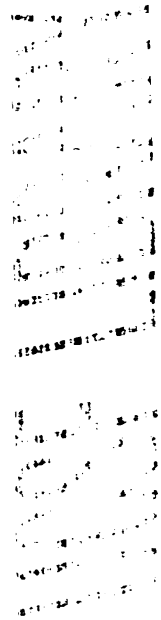


Figure 2: Brain Regions involved in drug self-administration or in learning associations. Areas pertinent to this thesis are denoted in color. NAcc= Nucleus Accumbens. VTA= ventral tegmental area. Amy= amygdala.

Chapter 1: Cholecystokinin

Introduction

Although the peptide cholecystokinin (CCK) was first isolated from the gut over a half-century ago (Ivy & Oldberg, 1928; Harper & Roper, 1943), its presence in the central nervous system was not established until the 1970's (Vanderhaegen, Signeau, & Gepts, 1975; Ljungdahl, Hokfelt, & Nilsson, 1978). CCK meets the criteria required for classification as a neurotransmitter, including neuronal synthesis, synaptic release, receptor specificity, and peptidase-dependent degradation (Crawley & Corwin, 1994). CCK has been shown to have effects on reproductive behavior, sleep, memory, anxiety, and analgesia (Crawley et al., 1994).



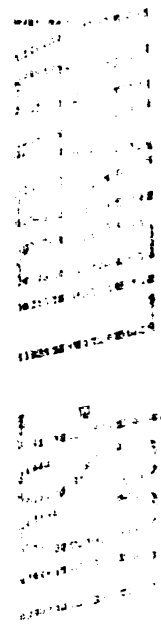
Two CCK receptor subtypes (CCK_A and CCK_B) have been characterized (Innis & Snyder, 1980; Moran, Robinson, Goldrich, & McHugh, 1986), and have been shown to be present in the central nervous system (Saito, Sankaran, Goldfine, & Williams, 1980; Saito, Goldfine, & Williams, 1981; Honda, Wada, Battey, & Wank, 1993; Shigeyoshi, Okamura, Inatomi, Matsui, Ito, Kaji, Abe, Nakata, Chiba, & Chihara, 1994). In the rat, mRNA for both the CCK_A and CCK_B receptor is expressed in the olfactory bulb, anterior olfactory nucleus and piriform cortex, hippocampus, basal ganglia, septum, interpeduncular nucleus, reticular thalamus, and layers II-VI of the cerebral cortex. In addition, CCK_A receptor mRNA is expressed in the paraventricular,

arcuate, and medial preoptic hypothalamus and CCK_B mRNA is expressed in the paraventricular thalamus and amygdala (Honda et al. 1993). The CCK-4 fragment binds with higher affinity to the CCK_B receptor while the CCK-8 fragment binds with higher affinity to the CCK_A receptor (Crawley et al., 1994).

In vitro activation of the CCK_A receptor produces a neuronal depolarization while activation of the CCK_B receptor produces a hyperpolarization (Branchereau, Bohme, Champagnat, Morin-Surun, Durieux, Blanchard, Roques, & Denavit-Saubie, 1992). Furthermore, microinjection of CCK_A agonists into the nucleus accumbens stimulates dopamine release while microinjection of CCK_B agonists into the nucleus accumbens suppresses dopamine release (Marshall, Barnes, Hughes, Woodruff, & Hunter, 1991). Therefore, it has been suggested that the CCK_A and CCK_B receptors have opposite cellular functions. Although the mechanisms responsible for the differences between CCK_A and CCK_B receptor effects are not yet apparent, in vitro studies have demonstrated that CCK can reduce potassium conductance, reverse μ receptor-mediated inhibition of calcium current, and increase conductance through chloride channels (Liu, Xu, Xu, Li, Yu, Kang, & Han, 1995; Yasui & Kawasaki, 1995; Shigeri, Shinohara, Murata, Fujimoto, & Kawasaki, 1996; Miller, Hoffer, Svoboda, & Lupica, 1997). When these data are taken together with evidence that CCK_A antagonists attenuate, while CCK_B antagonists potentiate, the reinforcing effects of morphine administration (Higgins, Nguyen, & Sellers, 1992), it becomes possible to speculate that the anti-opioid effects of CCK may be due, in part, to the ability

of CCK to reverse opioid-mediated changes in calcium and potassium conductance through action at CCK_B receptors.

There is a striking overlap in the distribution of CCK and opioid receptors in the CNS. CCK and enkephalin are both present in the RVM (Mantyh & Hunt, 1984; Skinner, Basbaum, & Fields, 1997) and are co-localized in neurons and axons in the rostral periaqueductal gray, medial lemniscus, central medial, paracentral, interanterodorsal, and ventral anterior thalamus, anterior olfactory nucleus, and in layers II and III of the neocortex (Gall, Lauterborn, Burks, & Seroogy, 1987). This suggests that both CCK and enkephalin are acting in brain regions known to be important in nociceptive modulation and that CCK may function to antagonize opioid analgesia by acting on the same cells or circuits that enkephalin acts on to produce analgesia.



CCK and serotonin are also co-localized in neuronal populations which contribute to the modulation of nociception, including a sub-population of ventral medulla neurons, some of which project to the dorsal horn of the spinal cord (Mantyh et al., 1984; Le Bars, 1988). CCK antagonizes μ and κ receptor-mediated opioid analgesia in the spinal cord (Wang, Wang, & Han, 1990), while serotonin, released from RVM axons, can contribute to spinal analgesia (Yaksh, 1979; Le Bars, 1988; Roberts, 1988). Furthermore, the CCK-mediated anti-analgesia produced by a conditioned safety signal can inhibit the analgesic effect of a serotonin agonist in the spinal cord (Watkins, McGorry, Schwartz, Sisk, Wiertelak, & Maier, 1997). This suggests

that both CCK and serotonin originating in the RVM contribute to the modulation of nociception at the level of the spinal cord.

CCK also modulates the effects of the neurotransmitter dopamine. CCK and dopamine are co-localized in a subset of VTA neurons that project to the posterior nucleus accumbens, medial septum, amygdala, and frontal cortex (Hokfelt, Skirboll, Rehfeld, Goldstein, Markey, & Dann, 1980; Fallon, Hicks, & Loughlin, 1983; Seroogy, Dangaran, Lim, Haycock, & Fallon, 1989). While CCK_A receptor activation potentiates dopamine release, activation of the CCK_B receptor attenuates dopamine release (Marshall, et al., 1991; Crawley, 1992; Ladurelle, Keller, Roques, & Dauge, 1993). CCK and dopamine interact at the level of the nucleus accumbens to potentiate the effects of conditioned reinforcers (Phillips, Le Noury, Wolterink, Donselaar-Wolterink, Robbins, & Everitt, 1993), while the analgesic effect of systemic morphine administration is reportedly antagonized by the microinjection of CCK into the nucleus accumbens (Pu, Zhuang, & Han, 1994). Additionally, the microinjection of an enkephalin analog into the VTA produces an increase in dopamine metabolites in the nucleus accumbens (Kalivas, Widerlov, Stanley, Bruse, & Prange, 1983) and ablation of striatal dopaminergic neurons or dopamine receptor blockade results in elevated levels of enkephalin in the nucleus accumbens (Hong, Yang, Gillin, & Costa, 1980; Tang, Costa, & Schwartz, 1983; Voorn, Roest, & Groenwegen, 1987). This suggests that CCK and dopamine interact in areas of the brain important in the modulation of learning and reward and that

they may also be interacting at sites that modulate the analgesic effects of opioids.

CCK and Anxiety

It is well-established that CCK contributes to anxiety in humans and in animal models. In humans, the i.v. administration of CCK-4 or CCK-5 produces symptoms identical to panic attack and these effects can be blocked by the administration of either a CCK_B receptor antagonist or the benzodiazepine lorazepam (Bradwejn, Koszycki, Shriqui, & Meterissian, 1990; Abelson, & Nesse, 1990; De Montigny, 1989; Bradwejn, Koszycki, Couetoux du Tertre, Megen, Boer, Westenberg, & Annable, 1994; Lines, Challendor, & Traub, 1995). In rodents, CCK increases the firing rate of pyramidal cells in the hippocampus, and this increase can be reversed by benzodiazepine administration (Bradwejn & De Montigny, 1984; 1985). Benzodiazepine treatment also decreases the responsiveness of hippocampal neurons to CCK (Bouthillier & De Montigny, 1988) and benzodiazepine withdrawal increases the number of CCK receptors in the frontal cortex and hippocampus (Harro, Lang, & Vasar, 1990). Furthermore, CCK_A receptor antagonists increase exploratory behavior in environments considered stressful or anxiety provoking, while the administration of CCK agonists have the opposite effect (Evans, 1986; Chang & Lotti, 1986; Harro, Pold, & Vasar, 1990; Hendrie, Neill, Shepherd, & Dourish, 1993). A negative correlation has also been demonstrated between performance on an anxiety test and number of CCK receptors in the frontal cortex. Rats that enter

the open arms of an elevated plus maze (and are therefore considered to be less anxious) display fewer CCK receptors in the frontal cortex (Harro, Kiivet, Lang, & Vasar, 1990).

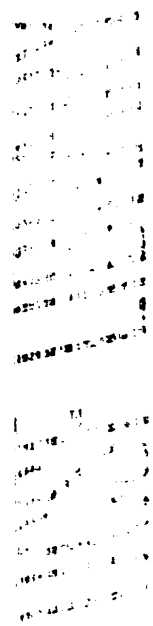
CCK and Analgesia:

CCK attenuates the antinociception induced by the administration of morphine, β-endorphin, and μ-receptor selective agonists, while CCK antagonists enhance the antinociception induced by these same compounds (Itoh, Katsuura, & Maeda, 1982; Faris, Komisaruk, Watkins, & Mayer, 1983; Watkins, Kinscheck, Kaufman, Miller, Frenk, & Mayer, 1985; Baber, Dourish, & Hill, 1989; Wang, Wang, & Han, 1990; Magnuson, Sullivan, Simonnet, Roques, & Dickenson, 1990; Valverde, Maldonado, Fournie-Zaluski, & Roques, 1994; Vanderah, Bernstein, Yamamura, Hruby, & Porecca, 1996). However, there is little evidence that CCK affects pain behaviors in the absence of an endogenous or exogenous opioid (Lavigne, Millington, & Mueller, 1992), suggesting that CCK is not hyperalgesic but, rather, anti-opioid in nature.

Effects of CCK on Tolerance and Withdrawal

Although CCK administration affects the acquisition of morphine tolerance (Tang, Chou, Iadarola, Yang, & Costa, 1984; Dourish, Hawley, & Iversen, 1988; Dourish, O'Neill, Coughlan, Kitchener, Hawley, & Iversen, 1990; Xu, Wiesenfeld-Hallin, Hughes, Horwell, & Hokfelt, 1992), CCK does not precipitate morphine withdrawal in morphine

dependent rats (Maldonado, Valverde, Derrien, Tejedor-Real, & Roques, 1994) and CCK antagonists do not modify either opioid self-administration or opioid discrimination (Higgins, Joharchi, Wang, Corrigall, & Sellers, 1994). However, CCK receptor antagonists differentially affect morphine place conditioning. Specifically, CCK_A receptor antagonists block the acquisition of morphine conditioned place preference while CCK_B receptor antagonists slightly potentiate morphine conditioned place preference (Higgins, et al., 1992).



Summary

The effects of CCK in the central nervous system are far-reaching. CCK can delay opioid tolerance and can act in a number of brain regions to antagonize analgesia. It is the intention of this thesis to further delineate the role of CCK in morphine-induced analgesia and morphine tolerance by assaying the effects of CCK microinjections into various brain nuclei in naive and morphine tolerant animals.

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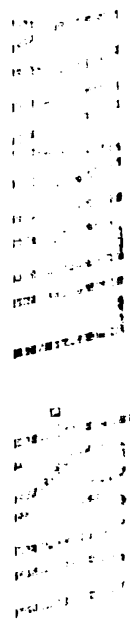

Chapter 2: Morphine

Introduction

Extracts from the opium poppy (*Papaver somniferum*) have been used in the treatment of pain for almost 3000 years (Jaffe & Martin, 1985; Fields, 1987). Morphine, one of the constituent alkaloids of opium, was first purified from opium extract in 1806 (Jaffe et al., 1985). The presence of an endogenous opioid and specific opioid binding site was suggested in the 1960's (Portoghese, 1965) but was not identified until almost a decade later (Pert & Snyder, 1973; Kuhar, Pert, & Snyder, 1973; Lowney, Schulz, Lowery, & Goldstein, 1974; Hughes, Smith, Kosterlitz, Fothergill, Morgan, & Morris, 1975). There are currently four known groups of endogenous opioid peptides: Leucine and methionine enkephalin, β-endorphin, dynorphin, and endomorphin (Fields & Basbaum, 1999). These endogenous peptides demonstrate moderate receptor selectivity for the three known opioid receptors implicated in pain modulation: the μ, δ, and κ receptor. The μ opioid receptor has the highest selectivity for endomorphin, the δ for the enkephalins, and the κ opioid receptor for dynorphin (Martin, Eades, Thompson, Huppler, & Gilbert, 1976; Lord, Waterfield, Hughes, & Kosterlitz, 1976; Chapman, Diaz, & Dickenson,1997).

The μ receptor is widely distributed in the central nervous system, with the highest concentrations of mRNA and immunohistochemical

staining being found in the thalamus, brainstem, nucleus accumbens, septum, hippocampus, amygdala, locus coeruleus, caudate-putamen, parabrachial nucleus, central gray, spinal cord and dorsal root ganglion (George, Zastawny, Briones-Urbina, Cheng, Nguyen, Heiber, Kouvelas, Chan, & O'Dowd, 1994; Mansour, Fox, Burke, Meng, Thompson, Akil, & Watson, 1994; Arvidsson, Riedl, Chakrabarti, Lee, Nakano, Dado, Loh, Law, Wessendorf, & Elde, 1995; Mansour, Fox, Burke, Akil, & Watson, 1995). mRNA and immunohistochemical staining for the δ receptor is primarily found in the cerebral cortex, olfactory tubercle, hippocampus, caudate-putamen, hypothalamus, nucleus accumbens, amygdala, red nucleus, spinal cord, and dorsal root ganglion (George et al., 1994; Mansour et al., 1994; Arvidsson, Dado, Riedl, Lee, Law, Loh, Elde, & Wesendorf, 1995), while the highest concentration of mRNA and immunohistochemical staining for the κ receptor are found in the cortex, amygdala, stria terminalis, caudate-putamen, olfactory tubercle, nucleus accumbens, hypothalamus, ventral tegmental area, hippocampus, parabrachial nucleus, trigeminal nucleus, spinal cord, and dorsal root ganglion (George et al., 1994; Mansour et al. 1994; Mansour, Burke, Pavlic, Akil, & Watson, 1996).

Activation of opioid receptors produces a potassium current-mediated hyperpolarization in hippocampal CA1, thalamic, trigeminal, supraoptic, periaqueductal gray, raphe magnus, and spinal cord neurons (Pan, Williams, & Osborne, 1990; Chieng & Christie, 1994; Inenaga, Nagamoto, Nakao, Yanihara, & Yamashita, 1994; Grudt & Williams, 1994; Brunton & Charpak, 1998;

Fujibayashi, Kubota, & Saito, 1998; Svoboda & Lupica, 1998). In addition, activation of opioid receptors produces an indirect decrease in an inward sodium current and changes in cAMP and in cAMP-dependent protein kinase activity (Nestler, Hope, & Widnell, 1993).

Morphine and Analgesia

Although morphine binds to δ and κ receptors, morphine analgesia is primarily dependent upon activation of the μ receptor (Fang, Fields, & Lee, 1986; Dickenson, 1991; Matthes, Maldonado, Simonin, Valverde, Slowe, Kitchen, Befort, Dierich, Le Meur, Dolle, Tzavara, Hanoune, Roques, & Kieffer, 1996). Morphine has been shown to produce analgesia in the rat tail flick and paw withdrawal tests (Yaksh & Rudy, 1976; Wang, 1977; Yaksh & Rudy, 1977; Yaksh, Wilson, Kaiko, & Inturrisi, 1979), and has been used clinically for the treatment of post-operative pain, cancer pain, bone pain, deep tissue pain, visceral pain, and certain types of neuropathic pain (Twycross, 1994). Morphine acts at multiple sites within the endogenous pain modulating system to produce its analgesic effects (Yeung, Yaksh, & Rudy, 1978; Yaksh & Noueihed, 1985), and the effects of morphine at these different sites are synergistic (Yeung & Rudy, 1980).

Morphine and Tolerance

Tolerance is the phenomenon by which repeated administration of a drug results in a decrease in effectiveness or in the need to take a larger dose of the drug to maintain the same effect. Tolerance to the

analgesic properties of opioids has been clearly demonstrated in animal models (Cochin & Kornetsky, 1964; Yaksh, 1991). However, the question of whether analgesic tolerance to morphine develops in the clinical setting is more controversial. While some researchers have noted a decrease in the analgesic effectiveness of opioids with repeated administrations (McQuay, Bullingham, & Moore, 1981; Wallenstein, Houde, Portenoy, Lapin, Rogers, & Foley, 1990; Gourlay, Plummer, Cherry, Onley, Parish, Wood, & Cousins, 1991), others have not observed a change in effectiveness of these drugs over time (Urban, France, Steinberger, Scott, & Maltbie, 1986; Inturissi, Portenoy, Max, Colburn, & Foley, 1990; Plummer, Cherry, Cousins, Gourlay, Onley, & Evans, 1991). Therefore, it has been proposed that chronic pain antagonizes the analgesic tolerance that would otherwise develop following repeated drug exposure (Colpaert, 1996). Additionally, while it has been established that patients with progressive diseases such as cancer require larger doses of opioids over time, it is often impossible to determine if the change in drug dose reflects a change in the intensity of pain or in tolerance to the drug (Kanner & Foley, 1981; Foley, 1989; Collin, Poulain, Gauvin-Piquard, Petit, Pichard-Leandri, 1993).

Control over drug administration may be an important factor in the development of tolerance. For example, tolerance has not been demonstrated under conditions in which patients are allowed to self-administer morphine to control post-operative pain. However, tolerance does occur if similar post-operative patients are given a continuous morphine infusion (Chapman & Harlan, 1989; Hill,

Chapman, Kornell, Sullivan, Saeger, & Benedetti, 1990). This implies that morphine tolerance can be affected by the patient's conscious awareness of, and control over, drug administration.

Associative versus Non-associative Tolerance

There are two well-characterized types of opioid tolerance: associative and non-associative (Figure 1). Non-associative tolerance typically results from the administration of large doses of a drug over a short period of time and in the absence of specific environmental cues predictive of drug administration (Tiffany & Maude-Griffin, 1988; Dafters & Odber, 1989). Non-associative tolerance is also transient, and μ receptor specific (McLaughlin, Dewey, & Fanselow, 1991; Carter & Tiffany, 1996). In contrast, associative tolerance requires repeated administration and can be produced by relatively small doses of a drug in the presence of specific contextual cues (Tiffany et al., 1988; Dafters et al., 1989). Additionally, associative morphine tolerance is long-lasting and not specific to μ receptors (McLaughlin et al., 1991; Carter et al., 1996). While associative tolerance is environmentally specific, non-associative tolerance is not dependent on environmental cues. In other words, an animal that is associatively tolerant to morphine will not display tolerance in an environment other than that paired with drug administration (Siegel, 1976).

Morphine and Addiction

Addiction is typically defined as compulsive drug use resulting from conditions of tolerance, sensitization, and dependence. Morphine is considered an addictive substance in humans (White, 1996). Morphine addiction appears to be the consequence of long-term changes in the central nervous system following repeated administration (Nestler, et al. 1993). Recently, it was reported that the expression of an allele for the dopamine D2 receptor is correlated with opioid addiction in humans (Comings, Muhleman, Ahn, Gysin, & Flanagan, 1994; Noble, Lawford, Ritchie, Young, & Zhang, 1998) and that the absence of the dopamine D2 receptor in mice attenuates the rewarding effects of morphine (Maldonado, Saiardi, Valverde, Samad, Roques, & Borelli, 1997). Additionally, certain rat strains have been found to self-administer drugs of abuse more readily than others (Nestler, 1993). Therefore, morphine addiction also appears to be influenced by genetic factors.

Addiction is a difficult concept to investigate in an animal model. However, self-administration and conditioned place preference paradigms have been used to investigate the motivated behaviors that typically correlate with addictive drug use in humans (Koob, Sanna, & Bloom, 1998). Cumulative evidence suggests that the endogenous reward pathway is an important contributor to the addiction that results from the administration of morphine and other drugs of abuse (Wise, 1987; Di Chiara & Imperato, 1988; Wise, 1989). Currently, it is not understood how the transition from occasional drug use to drug addiction occurs, nor is it known why

some animals and individuals are more susceptible to addiction than others (Wise, 1996).

Summary

Morphine and other opioids have been successfully used for the treatment of pain in both humans and animals. Tolerance develops in response to the repeated administration of morphine. There are two well-established forms of morphine tolerance: associative and non-associative. Morphine is self-administered in animals and can be addictive in humans. One objective of this thesis is to expand upon the literature by investigating the effects of CCK on systemically administered morphine. Additionally, this thesis attempts to determine the effects of selective CCK antagonists on associative and non-associative morphine tolerance.

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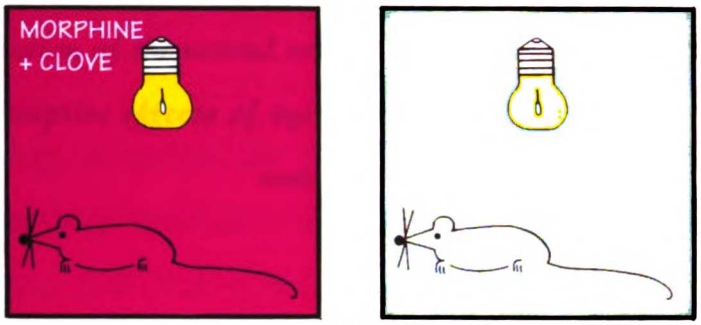

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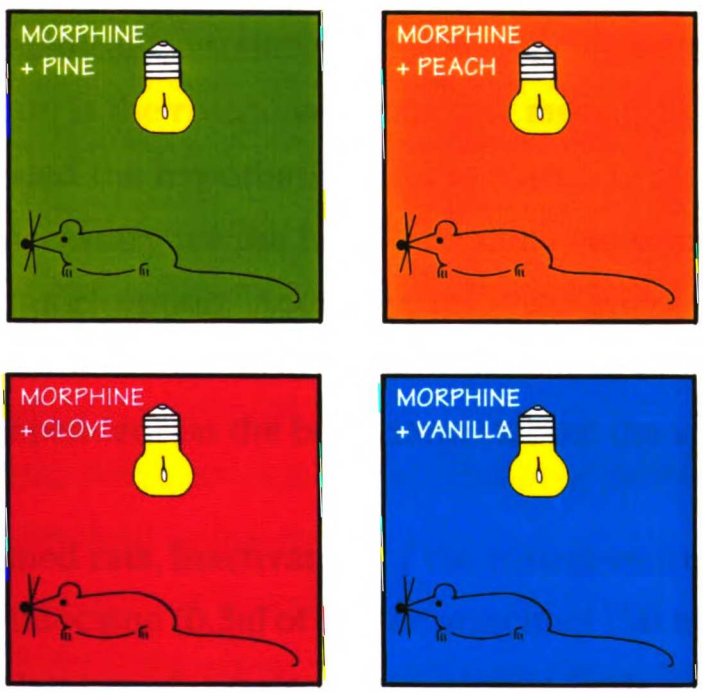

Figure 1: Associative (A) versus non-associative (NA) morphine tolerance. Associative tolerance is produced by repeatedly pairing a distinctive context with morphine administration while non-associative tolerance is produced by repeatedly administering a drug irrespective of environmental context. Associative tolerance is environmentally specific: animals will not be tolerant to morphine administered in a differen environment. Non-associative tolerancedoes not depend on environment: animals will be tolerant to morphine regardless of the environment in which it is administered.

Chapter 3:

The contribution of the rostral ventromedial medulla (RVM) to the antinociceptive effects of systemic morphine in restrained and unrestrained rats

Abstract

Although there are numerous opioid sensitive structures in the central nervous system, the contribution of each to the analgesic effect of systemically administered morphine is controversial. One such structure is the rostral ventromedial medulla. In the present study we tested the hypothesis that the rostral ventromedial medulla is necessary for the full expression of systemic morphine induced antinociception. Additionally, we examined whether the modulatory effect of the rostral ventromedial medulla on tail flick latency is dependent on the behavioral state of the animal.

In unrestrained rats, inactivation of the rostral ventromedial medulla with either lidocaine (0.5µl of 4%) or muscimol (50 ng) had no effect on tail flick latency. In contrast, in restrained rats, inactivation of the rostral ventromedial medulla with either lidocaine (0.5µl of 4%) or muscimol (50 ng) significantly decreased tail flick latency. In both conditions, microinjection of morphine (5 µg) into this region significantly increased tail flick latency. Additionally, in unrestrained rats, muscimol (50 ng) and CCK-4 (0.5 ng) infusion into the rostral ventromedial medulla completely reversed systemic morphine induced analgesia, while lidocaine (0.5 µl of 4%) and CCK-8

(0.25 ng) infusion partially reversed systemic morphine induced analgesia.

These findings demonstrate that the rostral ventromedial medulla does not tonically modulate tail flick latency in unrestrained rats, but does modulate tail flick latency when animals are stressed via restraint. These findings also strongly support the hypothesis that the rostral ventromedial medulla is necessary for the full analgesic effects of systemically administered morphine.

Introduction

Opioid agonists, such as morphine, are among the most effective pain relieving agents currently available. Despite significant progress in understanding their cellular and molecular mechanisms of action, the means by which systemically administered opioids relieve pain is still uncertain. Although it is well established that opioids can produce a profound analgesic effect through a direct action at the level of the spinal cord (Yaksh & Rudy, 1976; Yaksh, 1993), there is less agreement on the extent to which their direct actions at supraspinal structures contribute to systemic opioid analgesia. The current study addresses this issue by examining the contribution of the rostral ventromedial medulla (RVM) to tail flick latency under different behavioral conditions and following the administration of systemic morphine.

Among the targets for the supraspinal action of opioids in the production of analgesia is a circuit that includes the amygdala (AMY), the periaqueductal gray (PAG), and the rostral ventromedial medulla (RVM). Microinjection of opioid agonists into any one of these sites inhibits spinal withdrawal reflexes elicited by noxious stimuli (Pert & yaksh, 1974; Vanegas, Barbaro, & Fields, 1984; Helmstetter, Bellgowan, & Tershner, 1993). Both the AMY and the PAG project to the RVM (Basbaum & Fields, 1979; Magnuson & Gray, 1990; Price & Amaral, 1981) which, in turn, projects to the spinal cord dorsal horn laminae that receive direct input from primary afferent nociceptors and contain nociceptive projection neurons (Basbaum, Clanton, & Fields, 1978; Light & Perl, 1979; Ruda, Allen, & Gobel, 1981; Martin, Vertes, & Waltzer, 1985; Mason & Fields, 1989; Fields, Malick, & Burstein, 1995).

No significant direct projection from either the AMY or the PAG to the spinal cord dorsal horn has been identified (Fields & Basbaum, 1978; Hopkins & Helstege, 1978; Price et al., 1981; Holstege, 1988; Van Bockstaele, Aston-Jones, Pieribone, Ennis, & Shipley, 1991). Instead, the descending antinociceptive effects of the AMY and PAG are mediated via projections to RVM neurons (Basbaum & Fields, 1984; Kiefel, Rossi, & Bodnar, 1993; Roychowdhury & Fields, 1996). When the RVM is lesioned, the analgesia produced by the microinjection of opioids into either the AMY or PAG is blocked (Young, Watkins, & Mayer, 1984; Helmstetter, Tershner, Poore, & Bellgowan, 1996). Thus, through their actions on AMY, PAG, and

RVM neurons, opioids can produce antinociceptive effects at the level of the spinal cord.

Although the direct sensitivity of the RVM to the antinociceptive actions of opioids is undisputed, there is controversy about the contribution of this nucleus to the antinociceptive actions of systemically administered opioids. While some investigators have detected an immediate reversal of the antinociceptive effects of systemic morphine following microinjection of the opioid antagonist naloxone into the RVM (Dickenson, Oliveras, & Besson, 1979; Azami, Llewelyn, & Roberts, 1982), others have reported that RVM lesions have no clear effect on the antinociception induced by systemic opioids (Proudfit, 1980; Proudfit, 1981; Kelly & Franklin, 1984).

One possible explanation for these apparently contradictory results is that the effect of RVM inactivation is dependent on the behavioral state of the animal. Indeed, there is evidence which demonstrates that when an animal is experiencing stress or fear the results of RVM inactivation are substantially altered. For example, in comparison to unstressed animals, morphine has been shown to be more potent in animals subjected to restraint stress (Applebaum & Holtzman, 1984; Kelly et al., 1984; Calcagnetti, Fleetwood, & Holtzman, 1990). Furthermore, lesions of the RVM are reported to have no effect on morphine analgesia in unstressed animals (Kelly et al., 1984). In related work, several investigators have demonstrated that electrolytic lesions of the RVM can block the antinociceptive effects of aversive conditioned stimuli (Chance, 1980; Cannon, Lewis,

Weinberg, & Liebeskind, 1983; Watkins, Young, Kinscheck, & Mayer, 1983; Helmstetter & Tershner, 1994). Investigators have also found that while 5-HT (a neurotransmitter known to be involved in the descending control of nociception (Le Bars, 1988; Kiefel, Cooper, & Bodnar, 1992; Potrebic, Mason, & Fields, 1995)) is required for morphine analgesia in stressed animals, it does not appear to contribute to morphine analgesia in unstressed animals (Milne & Gamble, 1990; Kennett & Joseph, 1981). Additionally, since 5-HT is present in cell bodies and axon terminals throughout the RVM (Dahlstrom & Fuxe, 1964; Bowker, Westlund, & Coulter, 1981; Lovick & Robinson, 1983; Skagerberg & Bjorklund, 1985) and since the RVM is the major source of 5-HT in the dorsal horn of the spinal cord (Dahlstrom et al., 1964, Randic & Yu, 1976; Yaksh, 1979; Le Bars, 1988; Solomon & Gebhart, 1988), it may be critical to take the behavioral state of the animal into consideration when designing studies testing the influence of the RVM on nociception. Based on the evidence outlined above, it is reasonable to propose that the RVM mediates the interaction between stress and analgesia such that when an animal is experiencing stress or fear, the RVM is activated and contributes to analgesia.

One factor which has been implicated in the modulation of opioid analgesia is the neuropeptide cholecystokinin (CCK). This peptide has been shown to reduce or block opioid analgesia when administered systemically (Baber, Dourish, & Hill, 1989; Li & Han, 1989, Faris, Komisaruk, Watkins, & Mayer, 1983). CCK has been identified in RVM, PAG, and AMY axons and cell bodies (McDonald, 1985; Roberts,

Woodhams, Polak, & Crow, 1982; Gall, Lauterbron, Burks, & Seroogy, 1987; Liu, Chandler, Beitz, Shipley, & Behbehani, 1994; Mantyh & Hunt, 1984). Additionally, some CCK containing neurons in the RVM project to the spinal cord, and a subpopulation of CCK positive somata in the RVM contains 5-HT (Mantyh et al., 1984). Furthermore, stress induced analgesia is influenced by CCK. For example, conditioned fear produces an antinociceptive effect that involves the PAG-RVM pain modulatory circuit (Watkins, Cobelli, & Mayer, 1982; Watkins et al., 1983). This analgesic effect can be antagonized through a mechanism involving CCK (Wiertelak, Maier, & Watkins, 1992). Therefore, given the evidence outlined above, it is possible that CCK is an important modulator of the antinociceptive effect of endogenous opioids in the RVM.

The present studies were carried out to re-evaluate the contribution of the RVM to systemic opioid analgesia in stressed and unstressed animals and to investigate the possibility that CCK contributes to the stress-related modulation of systemic opioid analgesia through an action in the RVM. Tail flick latency was chosen as a means of measuring antinociception since the tail flick reflex is generally considered to be spinally mediated (Irwin, Houde, Bennett, Hendershot, & Seevers, 1950). Additionally, the use of a modified Hargreaves device made it possible to measure tail flick latencies from animals that were awake, unrestrained, and unstressed.

Materials and Methods

Subjects

Male Sprague-Dawley rats (Charles River, Wilmington, Massachusetts) weighing 300-325 grams at the time of surgery were housed two to a cage in hanging baskets on a Thoren rack. The colony room followed a standard 12 hour light-dark cycle with food and water available ad libitum. The temperature of the colony room was kept constant (21°C). Animals were tested at approximately the same time during their light cycle each day.

Surgery

Animals were anaesthetized with pentobarbital (50 mg/kg, i.p.) and placed in a stereotaxic frame. A 26 gauge stainless steel chronic guide cannula (Plastics One, Roanoke, VA) was implanted into the RVM along the midline (coordinates: -2.1 mm posterior to the interaural line and -8.5 mm ventral to the surface of the skull) and secured to the skull with dental cement. A stainless steel dummy cannula (Plastics One, Roanoke, VA) was inserted into the guide cannula and remained in place when the guide cannula was not in use. Body temperature was kept constant (38°C) during surgery with the aid of a circulating water pad. Animals were allowed to recover from surgery for at least one week prior to the initiation of the behavioral protocol. Following surgery, animals were administered a daily oral suspension of antibiotics in their drinking water.

Drugs

Sulfated cholecystokinin-8 (CCK-8), lidocaine hydrochloride, and muscimol hydrobromide were obtained from Research Biochemicals International (RBI), Natick, Massachusetts. Cholecystokinin tetrapeptide (CCK-4) was obtained from Peninsula Laboratories, Belmont, California. Morphine sulfate powder was obtained from the pharmacy at the University of California, San Francisco. All drugs were dissolved in distilled water. The osmolarity of each dissolved compound was measured and compared to a saline standard to ensure that microinjection would not cause hypotonicity (saline= 287 mOsm, morphine= 257 mOsm, CCK-4= 280 mOsm, CCK-8= 0 mOsm, lidocaine= 249 mOsm, muscimol= 281 mOsm). Drug doses were determined based on published reports of the physiologically relevant and selective doses of these compounds following i.c. microinjection (Martin, Papp, & Bacino, 1978; Crawley & Corwin, 1994; Urban & Smith, 1994).

Behavioral Apparatus

Tail flick measurements were performed using a modified Hargreaves device (Hargreaves, Dubner, Brown, Flores, & Joris, 1988). This device contained a radiant heat source with a steep, constant slope and a voltage-dependent holding temperature. The voltage could be adjusted, thereby producing a variety of response latencies for each animal. A photocell was positioned next to the Hargreaves' radiant heat source so that rapid vertical or horizontal movement of the tail terminated the trial. A cut-off time of 12 seconds was

utilized for the heat source to avoid tissue damage. The behavioral chamber used for testing procedures in Experiments 1 and 3 measured 38x38x38 inches and consisted of clear Plexiglas walls, a Plexiglas ceiling and a glass floor. The injection restrainer consisted of a white cotton sock and a piece of nylon elastic (6" x 8") stretched over a Plexiglas block and secured with Velcro fasteners. A clear acrylic restrainer (Fisher) measuring 6.75" x 1.88" was used to restrain animals during the testing procedures in Experiment 2. RVM injections were made using a 1µl Hamilton syringe (Hamilton Co., Reno, NV) attached to a 10 cm length of PE 50 tubing which, in turn, was connected to a 33 gauge injection cannula (Plastics One). Microinjections were conducted at a rate of 0.5µl per minute. Once inserted, injection cannulae extended 2mm past the end of the guide cannulae and were left in place for one minute following microinjections to minimize the flow of drug solution up the cannula track. The experimental room was lit with a 60 watt bulb.

Behavioral Protocol

Experiment 1

Animals were handled daily following surgery and were habituated to the injection restrainer and to the behavioral chamber for at least five days prior to testing. On the first day following surgery animals were exposed to an open cotton sock which was placed on the floor in front of them. Animals entered the sock and were allowed to remain in the sock for 5 minutes before being removed by the experimenter.

A small hole was cut in the sock over the animal's head to permit access to the cannula. On the second day, the animals again entered the sock and were then slipped under the nylon elastic of the injection restrainer. Animals remained in the injection restrainer for 5 minutes before being removed by the experimenter. Animals were then placed in the behavioral chamber for 30-90 minutes. This procedure was repeated up to the first testing day to ensure that animals were well habituated to the behavioral protocol.

On the first testing day, animals were habituated to the injection restrainer for 5 minutes before being placed in the behavioral chamber. While animals were in the injection restrainer their tails were blackened and marked at 2 cm intervals. Animals were allowed to habituate to the behavioral chamber for 15 minutes before a tail flick measurement was taken. The voltage on the heat lamp was adjusted for each animal to produce a stable 4-5 second tail flick latency. This voltage was used for the duration of the experiment. Tail flick latency was measured every 5 minutes for 45 minutes for a total of 9 tail flick measurements per animal. Tail flick measurements began at the base of the tail and then proceeded to the tip of the tail at each 2 cm marked division. Tail flick measurements then began again at the base of the tail. Animals were returned to their homecages following testing.

On the second, fourth, sixth, and eighth testing days, microinjections of saline (0.5 µl), lidocaine (0.5µl of 4%), muscimol (50 ng in 0.5 µl), and morphine (5 µg in 0.5 µl), respectively, were made into the RVM.

All microinjections were performed while animals were in the injection restrainer. Dummy cannulae were removed, an injection cannula was inserted and injections proceeded at a rate of 0.5 µl per minute. Dummy cannulae were then reinserted and animals were placed in the behavioral chamber and allowed to habituate for 15 minutes. Tail flick latency was measured as before.

Testing days three, five, and seven were used to measure baseline tail flick latencies and were identical in design to the first testing day.

Experiment 2

Animals were not handled following surgery nor were they habituated to either the injection restrainer or the clear acrylic restrainer prior to testing.

On the first testing day, animals were habituated to the injection restrainer for 5 minutes before being removed and placed in clear acrylic restrainers. While animals were in the injection restrainer their tails were blackened and marked at 2 cm intervals. Animals were allowed to habituate to the clear acrylic restrainers for 15 minutes before a tail flick measurement was taken. In order to compare drug effects between restrained and unrestrained animals, the voltage on the heat lamp was adjusted and tail flick latency was measured as in Experiment 1. Thus, animals in Experiment 2 had identical baseline tail flick latencies as animals in Experiment 1, even

though animals in Experiment 2 tended to require a higher voltage. Animals were returned to their homecages following testing.

On the second, fourth, sixth, and eighth testing days, microinjections of saline (0.5 µl), lidocaine (0.5µl of 4%), muscimol (50 ng in 0.5 µl), and morphine (5 µg in 0.5 µl), respectively, were made into the RVM. All microinjections were performed while animals were in the injection restrainer. Dummy cannulae were removed, an injection cannula was inserted and injections proceeded at a rate of 0.5 µl per minute. Dummy cannulae were then reinserted and animals were placed in the clear acrylic restrainers and allowed to habituate for 15 minutes. Tail flick latency was measured as before.

Testing days three, five, and seven were used to measure baseline tail flick latencies and were identical in design to the first testing day.

Experiment 3

Animals were handled daily following surgery and were habituated to the behavioral protocol as in Experiment 1.

On the first testing day, animals were habituated to the injection restrainer for 5 minutes before being placed in the behavioral chamber. While the animals were in the injection restrainer, their tails were blackened and marked at 2 cm intervals. Animals were allowed to habituate to the chamber for 15 minutes before a tail flick

measurement was taken. The voltage on the Hargreaves device was then adjusted for each animal as in Experiment 1. Tail flick latency was measured every 4 minutes for 40 minutes for a total of 10 measurements per animal. Two measurements were taken from each of the 5 divisions of the tail. Animals were then returned to their homecages.

On the second testing day, animals were administered 4 mg/kg morphine i.p. before being placed in the injection restrainer for 5 minutes. Animals were then placed in the behavioral chamber and allowed to habituate for 15 minutes. Tail flick latency was measured every 4 minutes for 40 minutes for a total of 10 measurements per animal. Two measurements were taken from each of the 5 divisions of the tail. Animals were removed from the behavioral chamber and placed in the injection restrainer. Dummy cannulae were removed, an injection cannula was inserted, and one of the following five drugs was microinjected into the RVM: muscimol (50 ng in 0.5µl), lidocaine (0.5µl of 4%), CCK-8 (.25ng in 0.5 µl), CCK-4 (0.5ng in 0.5µl), or saline (0.5 µl). Dummy cannulae were re-inserted and animals were placed back in the behavioral chamber and allowed to re-habituate for 15 minutes. Tail flick latency was measured as before. Animals were then returned to their homecages.

Testing days 3 and 4 were identical in design to testing days 1 and 2, respectively. Drug administration on testing days 2 and 4 was randomized and presented in a counter-balanced fashion. Each

animal received a total of two microinjections into the RVM. Animals never received the same drug twice.

Histology

At the conclusion of the experiment, animals were deeply anaesthetized with pentobarbital (150 mg/kg, i.p.), injected with 1μl pontamine sky blue (PSB), and perfused intracardially through the ascending aorta with 0.1 M phosphate buffered saline followed by 10% formalin. Brains were blocked and sectioned coronally at 75 μm on a freezing microtome. Sections were mounted on glass slides and stained with neutral red or cresyl violet. Lesion sites were identified using the atlases of Paxinos and Watson (1986) and Kruger, Saporta, & Swanson (1995). Three criteria were used to establish the center of the injection site. First, the area with the highest density of PSB marked the center of the injection site. Second, gliosis and tissue damage were apparent around the center of the injection site. Third, the tip of the 33 gauge injection cannula often created a small hole at the center of the injection site.

Statistical Analysis

The means of the 9 tail flick latency measurements in Experiments 1 and 2 and the 10 tail flick latency measurements in Experiment 3 were calculated for each animal in each test condition. Within each experiment, the means of the conditions were compared using Friedman one-way nonparametric ANOVAs followed by Wilcoxon

signed-rank tests. The means of the conditions were compared between experiments using Mann-Whitney U tests. A $p<.05$ was considered statistically significant. Since the mean latencies on the baseline days were not significantly different within any of the experiments (pairwise comparison) they were collapsed within each experiment for statistical analysis. Non-parametric statistics were employed for statistical analysis since the data was not normally distributed. All statistical tests were conducted using GB-STAT, version 5.1.2. All figures were created using Stat View, version 4.0, and Adobe Illustrator, version 5.5.

Results

Experiment 1

RVM and control injection sites are illustrated in Figure 1A.

In unrestrained animals the lidocaine, muscimol, and saline conditions were not significantly different from the baseline condition (Figure 3). In other words, the infusion of lidocaine (0.5 µl of 4%), muscimol (50 ng in 0.5 µl), or saline (1 µl) into the RVM did not result in a significant change in tail flick latency. However, the infusion of morphine into the RVM resulted in a significant increase in tail flick latency compared to all other conditions ($p<.05$). Additionally, the muscimol condition was significantly different from the lidocaine condition ($p<.05$) but was not significantly different from the saline condition. Control injections into areas outside of the

RVM did not result in a significant change in tail flick latency. As illustrated in Figure 2, baseline tail flick latency in awake, unrestrained animals remained stable over the duration of experimental day 1, as well as over the duration of all four experimental days on which baseline tail flick latencies were measured. Taken together, these findings indicate that in awake, unstressed animals reversible inactivation of the RVM does not affect tail flick latency as measured with the Hargreaves device but that microinjection of morphine into the RVM results in analgesia.

Since reversible inactivation of the RVM did not result in a change in tail flick latency, we hypothesized that in awake, unrestrained, unstressed animals the RVM does not tonically modulate tail flick latency, but that it becomes activated when animals experience either stress or anxiety, or following the administration of systemic opioids. Experiment 2 was designed to test this hypothesis.

Experiment 2

RVM and control injection sites are illustrated in Figure 1B. In restrained animals the lidocaine, muscimol, and morphine conditions were significantly different from the baseline and saline conditions and were also significantly different from each other (Figure 3, $p<.05$). Specifically, the infusion of either lidocaine (0.5 µl of 4%) or muscimol (50 ng in 0.5 µl) into the RVM significantly decreased tail flick latency while the infusion of morphine (5 µg in 0.5 µl) into the RVM significantly increased tail flick latency. The infusion of

muscimol into the RVM resulted in a significantly greater decrease in tail flick latency than did the infusion of lidocaine into the RVM ($p<.05$). A comparison of the muscimol and lidocaine conditions between stressed and non-stressed animals is reported in Figure 4. As this figure demonstrates, there was a significant difference ($p<.05$) in the effects of muscimol and lidocaine in stressed versus non-stressed rats. These findings indicate that, in contrast to the results seen above in non-stressed animals, in awake, stressed animals the reversible inactivation of the RVM with either muscimol or lidocaine significantly decreases tail flick latency. These findings confirm that the RVM modulates tail flick latency when animals are experiencing either stress or anxiety.

Experiment 3

RVM and control injection sites are illustrated in Figure 1C.

As can be seen in Figure 5B, the infusion of muscimol (50 ng in 0.5 µl) into the RVM resulted in a significant attenuation of the analgesic effects of systemic morphine in unrestrained rats ($p<.05$). Following the administration of muscimol, the mean tail flick latency was no longer significantly different from baseline. Therefore, muscimol infused into the RVM results in a complete reversal of the systemic effects of morphine.

Lidocaine (0.5µl of 4%) also resulted in a significant attenuation of the analgesic effects of systemic morphine ($p<.05$). This is illustrated

in Figure 5A. However, in the lidocaine condition tail flick latency remained significantly different from baseline ($p<.05$), indicating that lidocaine produced only a partial reversal of the effects of systemic morphine.

As demonstrated in Figure 5C, CCK-4 (0.5 ng in 0.5μl) infused into the RVM resulted in a significant attenuation of the analgesic effects of systemic morphine ($p<.05$). Following the administration of CCK-4, the mean tail flick latency was not significantly different from baseline. Thus, CCK-4 infused into the RVM results in a complete reversal of the systemic effects of morphine. CCK-8 (0.25 ng in 0 .5 μl) also significantly attenuated the effects of systemic morphine ($p<.05$). This is illustrated in Figure 5D. Following the administration of CCK-8, the mean tail flick latency was still significantly different from baseline ($p<.05$). Therefore, as with lidocaine, the infusion of CCK-8 into the RVM results in a partial reversal of the effects of systemic morphine.

As shown in Figure 5E, there was no significant difference in tail flick latency between the morphine conditions before and after the infusion of saline into the RVM. This suggests that the results reported above are not due to possible volume effects of drugs infused into the RVM or to a decrease in the effectiveness of the systemic morphine over time. Neither lidocaine, muscimol, CCK-4, nor CCK-8 significantly attenuated the effects of systemic morphine when administered into brainstem sites outside of the RVM. This

demonstrates that the drug effects reported above are due to actions within the RVM and not to surrounding medullary nuclei.

Discussion

There are three main findings of this study. First, our results confirm that under conditions of stress, RVM inactivation decreases tail flick latency. This finding emphasizes the importance of using an instrument such as the Hargreaves device to measure tail flick latency in awake, unrestrained animals. Under the conditions of this experiment, baseline tail flick latencies were observed to be stable over a period of several days. This is a critical advance because it allows for the measurement of the tail flick (a spinal reflex that has been used extensively to assess opioid analgesia and descending pain modulating circuits) in awake, unrestrained rats. Second, reversible inactivation of the RVM in awake, unrestrained, unstressed animals does not alter baseline tail flick latency. Therefore, under these conditions, the RVM does not exert a net tonic effect on the tail flick reflex. Third, the antinociceptive effect of systemically administered morphine in awake, unrestrained, unstressed animals is attenuated by the reversible inactivation of the RVM by lidocaine or muscimol, or by the microinjection of CCK receptor agonists into the RVM. These results provide the first definitive evidence that RVM neurons are necessary for the full antinociceptive effects of systemic opioids in unanesthetized, unrestrained rats.

The current results help resolve the controversy surrounding the effects of RVM lesions on baseline tail flick latency. As reported by Young et al. (1984), we found that RVM inactivation does not affect baseline tail flick latency in habituated, unstressed rats. In contrast, others have reported significant decreases in baseline tail flick latency following RVM inactivation (Proudfit, 1980; Proudfit, 1981; Lovick, 1985; Morgan & Fields, 1994; Urban et al., 1994), an effect which we observed only when testing rats under restraint. Since those studies which noted a decrease in tail flick latency following RVM inactivation were conducted in either anesthetized or restrained rats, it is likely that the differences in the effect of RVM inactivation are dependent on behavioral state.

There are also conflicting reports regarding the effect of RVM inactivation on the antinociception induced by systemic opioids. Although Proudfit (1981) reported that, in awake rats, RVM lesions had no effect on the inhibition of tail flick latency by systemic opioids when tested seven or more days post-lesion, Young et al. (1984) demonstrated a partial reversal of the inhibition of tail flick latency five days post-lesion, and Yaksh et al. (1977) observed a complete reversal at 14 days post-lesion. Additionally, both Proudfit (1981) and Young et al. (1984) observed an increase in the effectiveness of RVM lesions at blocking systemic morphine analgesia over a period of 10 to 21 days post-lesion. In contrast, Kelly and Franklin (1984) did not observe a change in the analgesic effectiveness of systemic morphine when animals were tested 21 days post-lesion. Our demonstration that reversible inactivation of

the RVM causes immediate and virtually complete reversal of morphine antinociception confirms and extends the findings of Young et al. (1984) and Yaksh et al. (1977) by illustrating that neither central reorganization nor inactivation of fibers of passage within the RVM are necessary to reverse the antinociceptive effects of systemic opioids. Additionally, the fact that muscimol was as effective as lidocaine at reversing opioid induced antinociception demonstrates that inactivation of RVM neurons and not fibers of passage is responsible. Our results indicate that RVM neurons are necessary for the antinociceptive effects of systemic opioids in awake, unstressed rats.

It is important to consider why Proudfit (1981) and Kelly and Franklin (1984) did not observe an immediate reversal of systemic morphine induced antinociception following nucleus raphe magnus (NRM) lesions and why Young et al. (1984) found only a partial reversal of systemic morphine antinociception following RVM lesions. Many of the lesions made by Proudfit and by Kelly and Franklin were restricted to a small section of the RVM (the NRM) as compared to the complete inactivation of the RVM which we utilized. Perhaps if larger RVM lesions had been made in these studies, a reversal of systemic opioid induced antinociception would have been observed. Indeed, when Young et al. restricted their lesions to the NRM, they also failed to observe an immediate reversal of systemic opioid antinociception. Their larger lesions, which included substantially more of the RVM, did result in immediate and significant reversal of the antinociception produced by systemic opioids.

There is a large body of evidence indicating that the enhancement of morphine analgesia by stress involves the RVM. For example, the antinociception associated with conditioned fear or forepaw shock can be reversed by lesioning the RVM (Chance 1980; Cannon et al., 1983; Watkins et al., 1983; Helmstetter et al., 1996). Furthermore, Kelly and Franklin (1984) have shown that stress potentiates morphine analgesia and that this potentiation is reversed by RVM lesions. This suggests that the magnitude of the RVM contribution to analgesia depends on the type of stress the animal experiences. Our results are consistent with the hypothesis that the contribution of the RVM to antinociception is determined by the behavioral state of the animal.

Any neuronal population which tonically inhibits tail flick latency can have a permissive effect on systemic morphine analgesia, and the inactivation of such neurons will reduce morphine's antinociceptive effect regardless of whether the activity of the neurons is affected by morphine. Conversely, manipulations that decrease the tail flick latency will shift the morphine dose-response curve to the right, thereby making it difficult to determine whether the contribution of a nucleus to systemic morphine antinociception is necessary or permissive (Levine, Murphy, Seidenwurm, Cortez, & Fields, 1980). While RVM inactivation decreases baseline tail flick latency in both anesthetized and awake, restrained rats, the present experiments demonstrate that in awake, unrestrained rats there is no net tonic tail flick inhibition originating in the RVM. Thus, the fact

that inactivating RVM neurons in this condition reverses the antinociceptive effect of systemic opioids shows that the RVM is necessary for this effect, not simply permissive.

The reversal of morphine antinociception we observed following the injection of CCK into the RVM is interesting in light of recent research which indicates that CCK can mediate the reversal of conditioned analgesia at the level of the spinal cord (Wiertelak et al., 1992), that systemically administered CCK antagonists have different effects on morphine induced antinociception in stressed versus non-stressed animals (Lavigne, Millington, & Mueller, 1992), and that systemic administration of CCK to human subjects can produce symptoms of panic and anxiety (Lines, Challenor, & Traub, 1995). Additionally, since 5-HT modulates morphine analgesia in stressed animals, and since 5-HT and CCK are co-localized within the RVM (Mantyh et al., 1984) it is possible that CCK modulates the neural circuits involved in stress and fear, as well as having anti-analgesic actions, at the level of the RVM. However, our results indicate that stress is not required in order for CCK to exert an anti-analgesic effect at the level of the RVM.

There are currently two identified CCK receptors, the CCK-A receptor and the CCK-B receptor (Crawley & Corwin, 1994). Since CCK-4, which binds with higher affinity to the CCK-B receptor, was more effective at reversing morphine induced analgesia than CCK-8, which binds with higher affinity to the CCK-A receptor, our results suggest that the CCK-B receptor may be responsible for mediating the

reversal of systemic morphine induced analgesia at the level of the RVM.

CONCLUSIONS

In summary, our findings, when taken in conjunction with the findings of others, suggest that the contribution of the RVM to nociceptive responsiveness varies with the behavioral state of the animal. Any study of the contribution of the RVM to the effect of systemic opioids must, therefore, take into account the behavioral state of the animal. In animals not exposed to a known stressor, such as restraint, inactivation of the RVM had no effect on baseline tail flick latency, indicating that there was no tonic RVM control of this spinal reflex. However, RVM inactivation still significantly reduced the analgesic effect of systemic opioids, demonstrating that the RVM contribution to systemic opioid antinociception is necessary even in the absence of stress.

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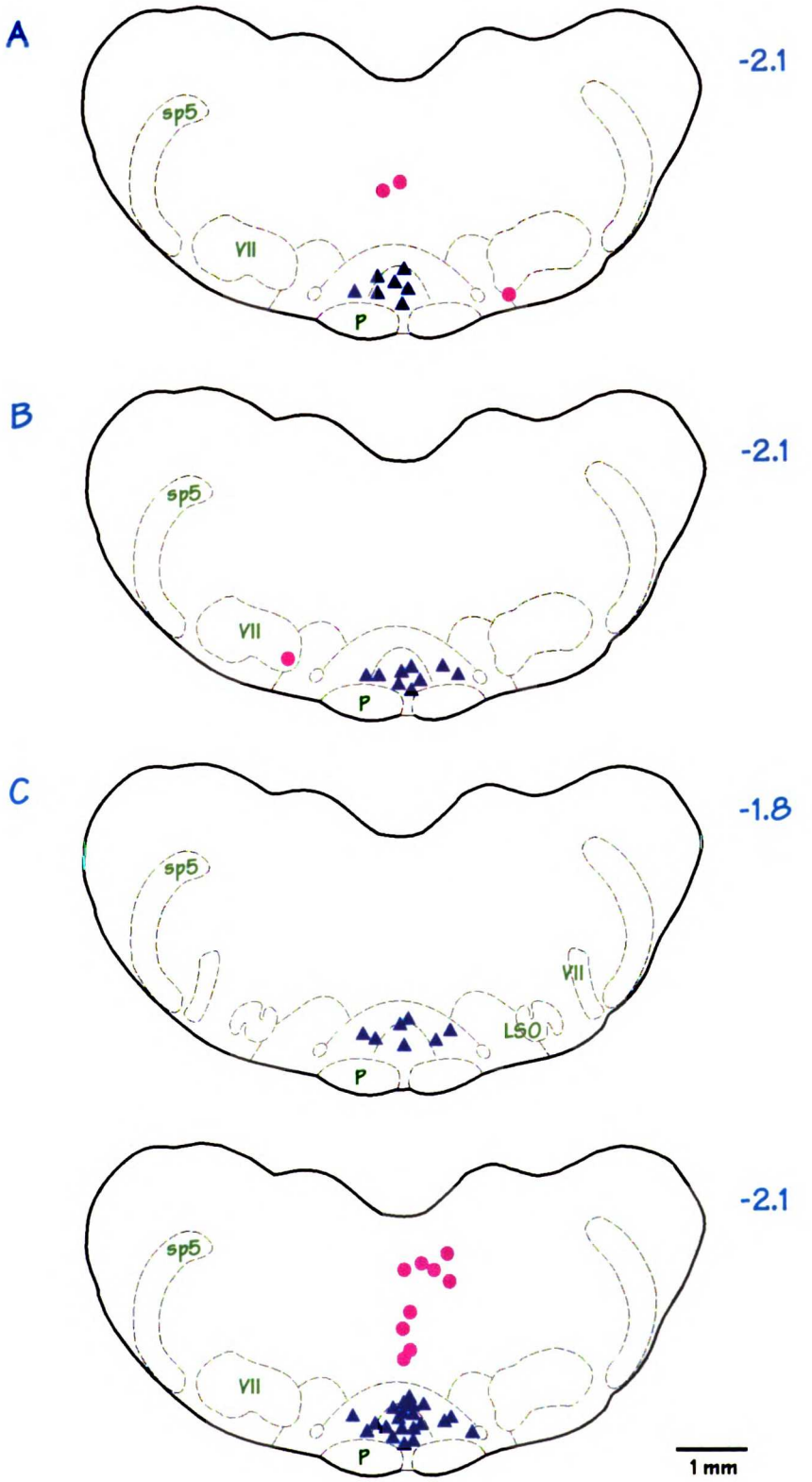


Figure 1: A) injection sites for experiment 1. B) injection sites for experiment 2. C) injection sites for experiment 3. ▲ = injection site contained within the RVM. ● = injection site outside of the RVM. Coordinates to the right of the figure refer to distance posterior to the interaural line. VII = facial nucleus, P = pyramidal tract, sp5 = spinal trigeminal tract, & LSO = lateral superior olive.

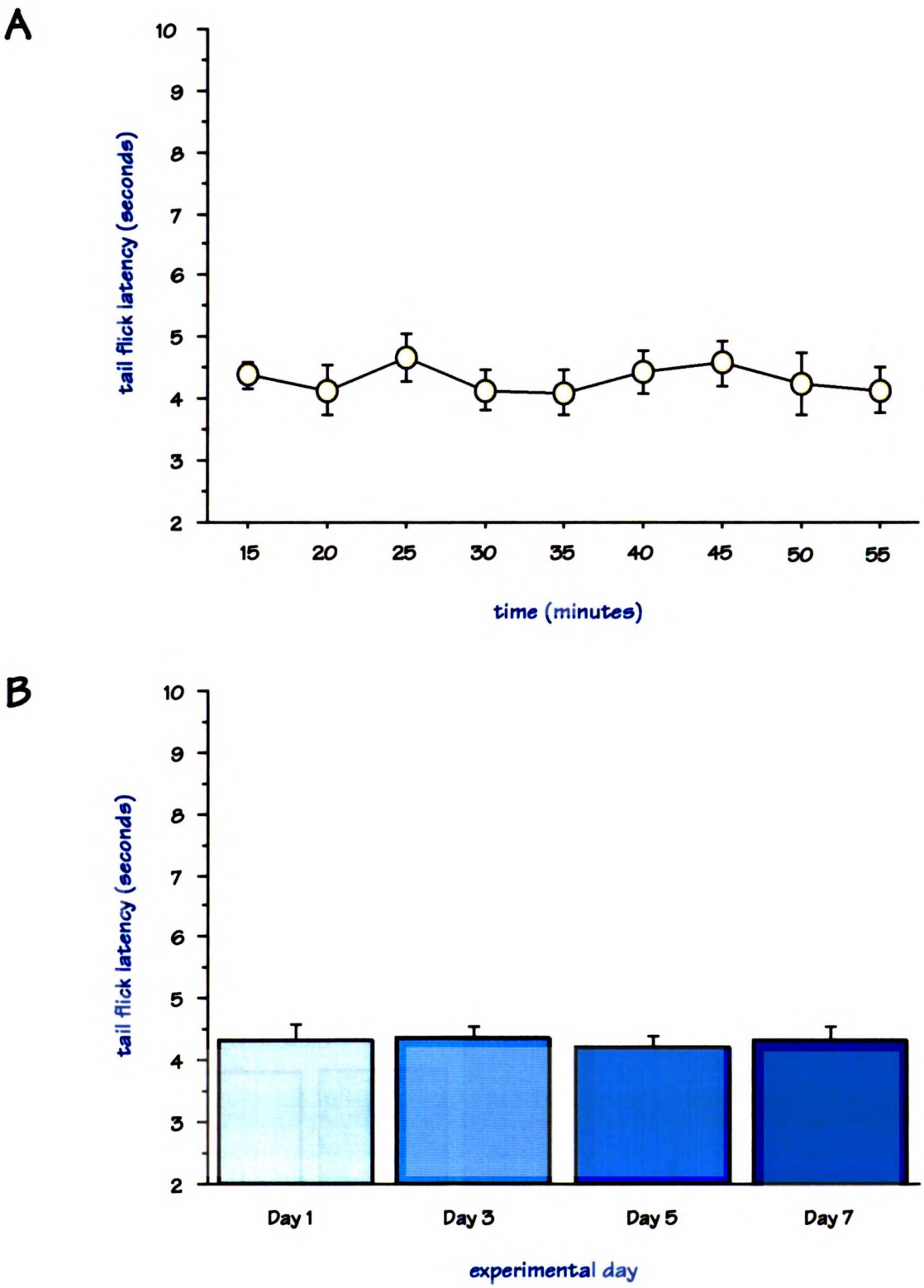


Figure 2: Stability of tail flick latency in unrestrained rats using the Hargreave's device. A) mean baseline latency for nine consecutive tail flick measurements made per animal on experimental day 1 (n=7). B) mean tail flick latency for each of four baseline days (experimental day 1, day 3, day 5, and day 7; n=7). Error bars represent standard error of the means. Tail flick latency is measured in seconds.

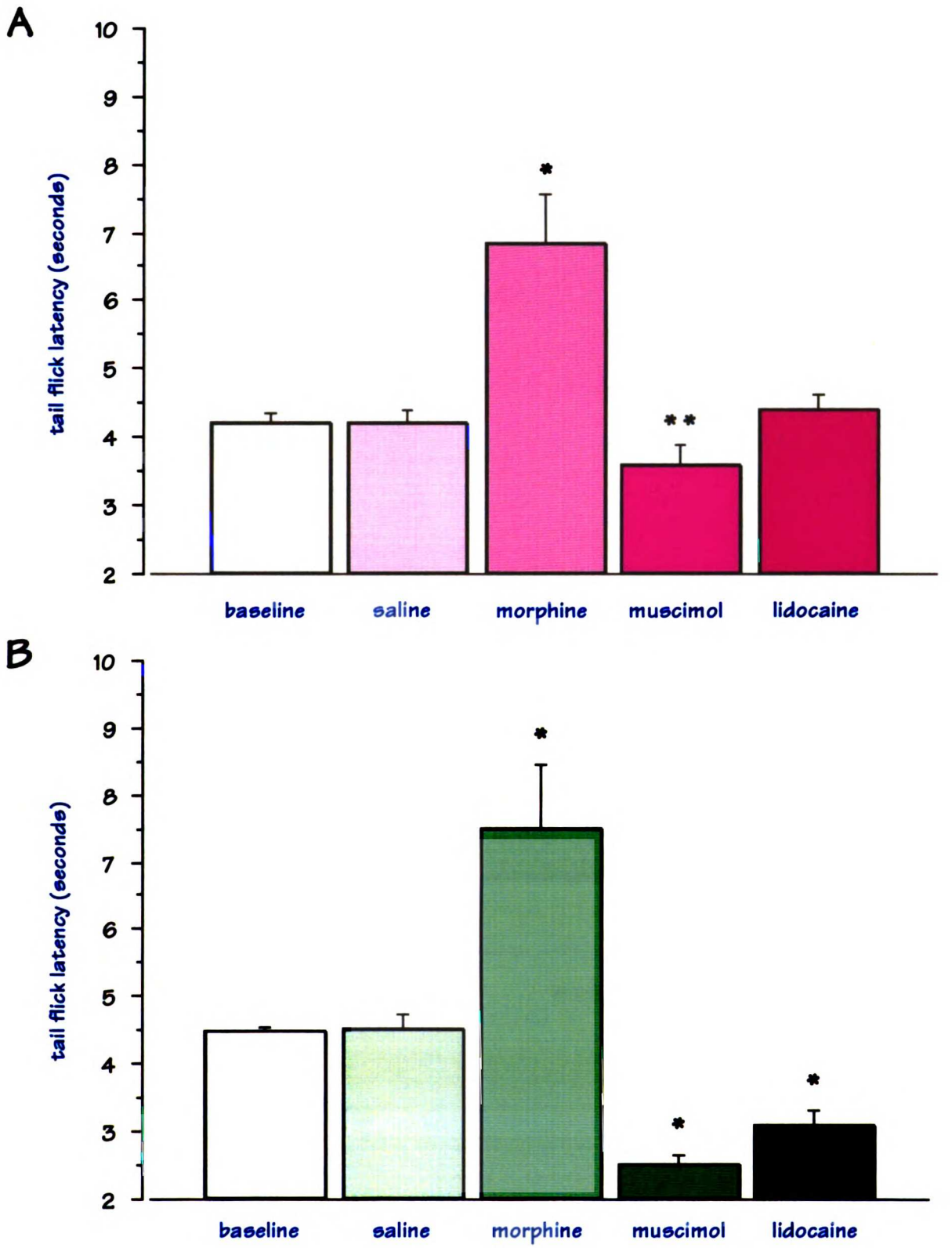


Figure 3: tail flick latency (measured in seconds) following microinjection of saline (0.5 µl), lidocaine (0.5 µl of 4%), muscimol (50ng in 0.5 µl), or morphine (5 µg in 0.5 µl) into the RVM of unrestrained (A: n=7) or restrained (B: n=9) animals. Error bars represent standard error of the means. * = significantly different from all other groups. $p<.05$. ** = significantly different from lidocaine only. $p<.05$.

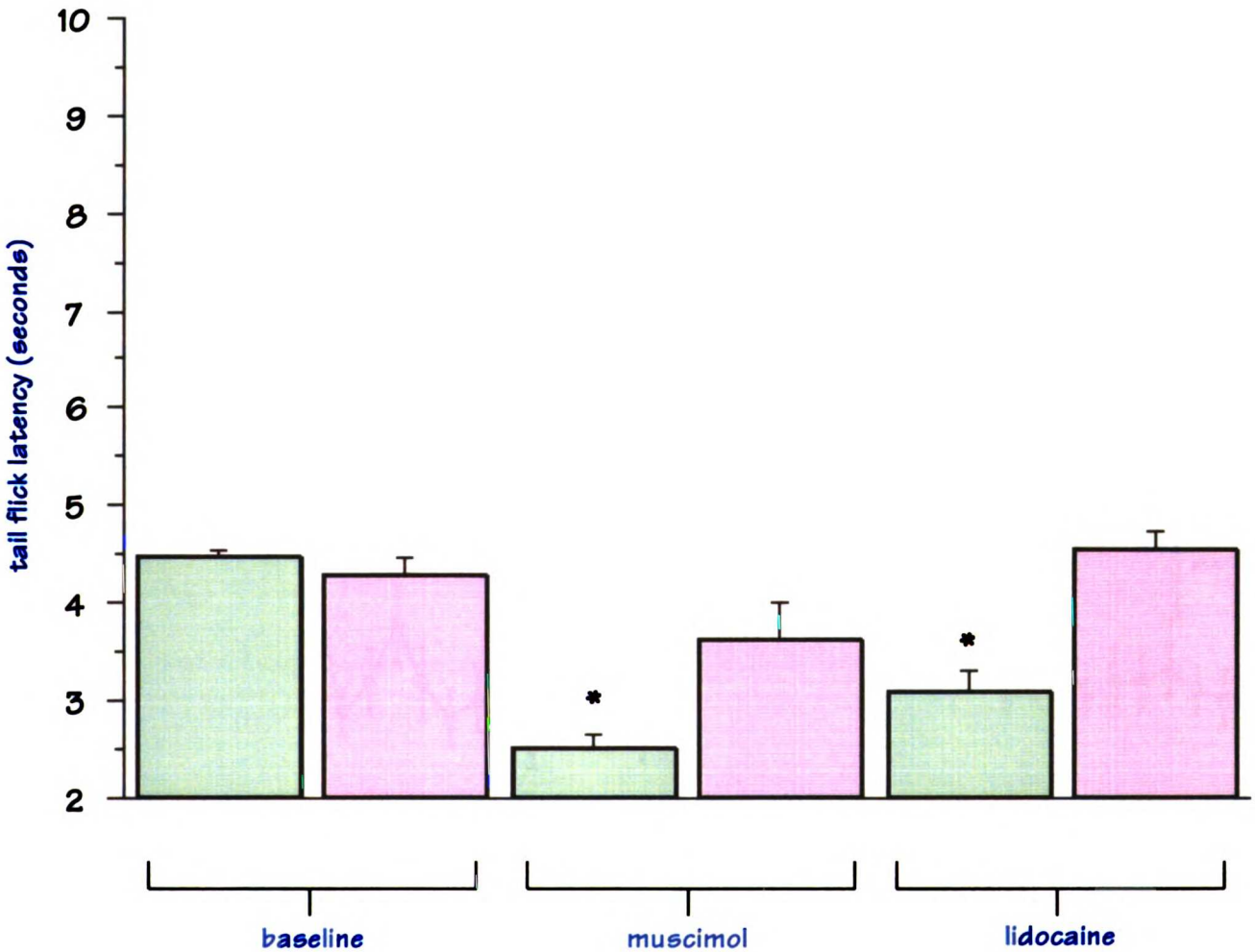


Figure 4: tail flick latency following the injection of either muscimol (50 ng in 0.5µl) or lidocaine (0.5µl of 4%) in restrained (green bars: n=9) or unrestrained (pink bars: n=7) animals. Error bars represent standard error of the means. * = significantly different from all unrestrained conditions. $p<.05$.

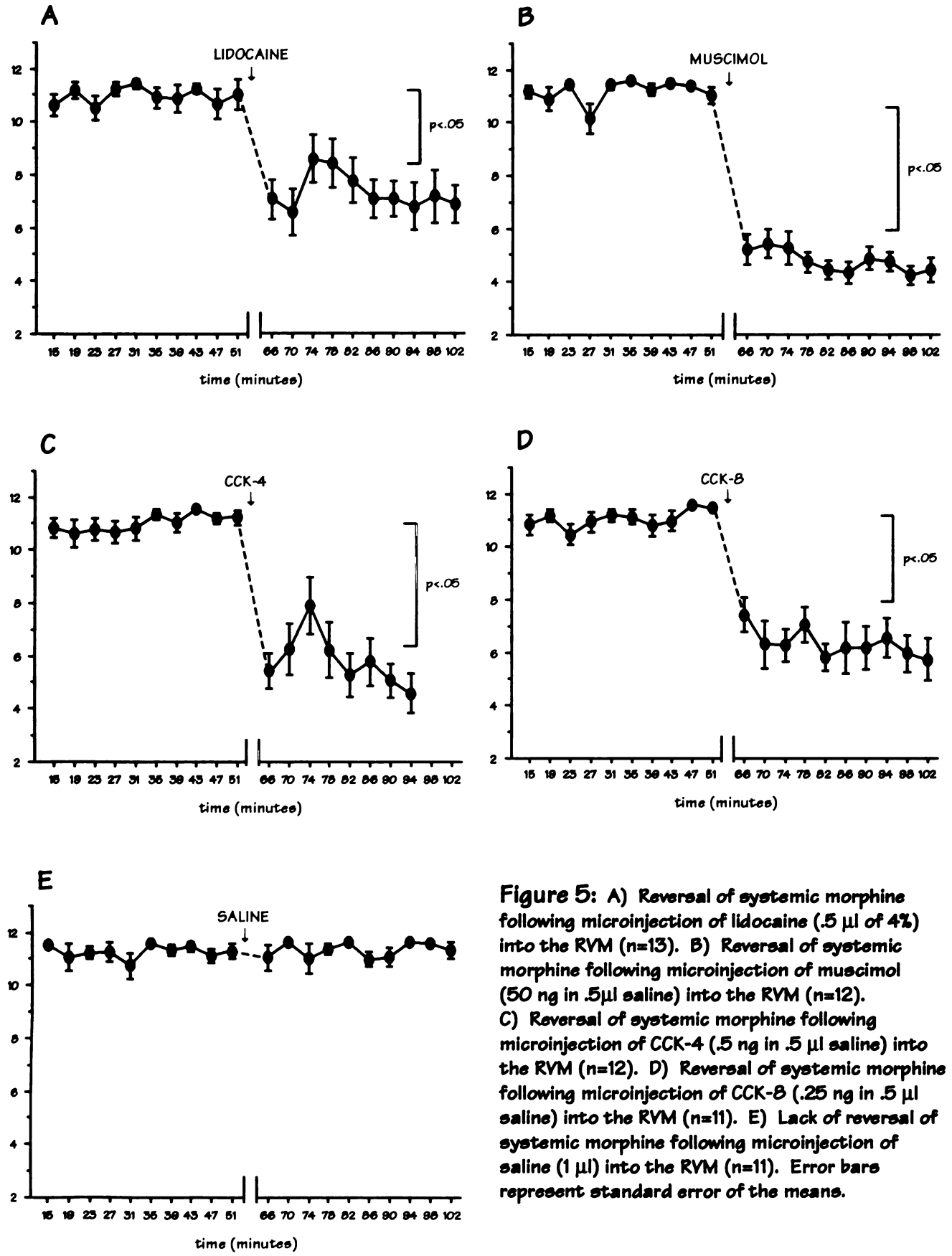


Figure 5: A) Reversal of systemic morphine following microinjection of lidocaine (.5 µl of 4%) into the RVM (n=13). B) Reversal of systemic morphine following microinjection of muscimol (50 ng in .5µl saline) into the RVM (n=12). C) Reversal of systemic morphine following microinjection of CCK-4 (.5 ng in .5 µl saline) into the RVM (n=12). D) Reversal of systemic morphine following microinjection of CCK-8 (.25 ng in .5 µl saline) into the RVM (n=11). E) Lack of reversal of systemic morphine following microinjection of saline (1 µl) into the RVM (n=11). Error bars represent standard error of the means.

Chapter 4:

A CCK-B receptor antagonist reverses associative but not non-associative morphine analgesic tolerance in the rat.

Abstract

There are two distinct types of opioid tolerance: associative (conditioned) and non-associative (non-conditioned). Associative tolerance is produced by repeatedly pairing a distinctive context with opioid administration, while non-associative tolerance is produced by administering an opioid unpaired with a distinctive context (Siegel, 1976; Siegel, Hinson, Krank, & McCully, 1982; Siegel & MacRae, 1984; Ehrman, Ternes, O'Brien, & McLellan, 1992). These two types of tolerance involve different drug doses and routes of administration (Dafters & Odber, 1989), different interdose intervals (McLaughlin, Dewey, & Fanselow, 1991; Tiffany & Maude-Griffin, 1988), and different retention periods following drug abstinence (McLaughlin et al., 1991; Dafters et al., 1989). Additionally, while non-associative morphine tolerance has been shown to be μ opioid receptor specific, associative morphine tolerance involves both κ and μ opioid receptors (Carter & Tiffany, 1996). Both types of tolerance have been postulated to result from the production of a compensatory response which acts to lower the effectiveness of opioids upon subsequent administrations. While many factors have been implicated in the acquisition and expression of non-associative tolerance, a biological correlate for associative tolerance has yet to be identified. Here we demonstrate that cholecystokinin (an anti-opioid

peptide) is required for associative but not for non-associative tolerance to morphine. Additionally, we demonstrate that this effect is due to action at the CCK-B receptor.

Introduction

The role of cholecystokinin (CCK) as an anti-opioid peptide is firmly established. CCK suppresses the analgesia induced by the administration of morphine, β-endorphin, and μ-receptor agonists (Itoh, Katsuura, & Maeda, 1982; Faris, Komisaruk, Watkins, & Mayer, 1983; Baber, Dourish, & Hill, 1989; Magnuson, Sullivan, Simonnet, Roques, & Dickenson, 1990). CCK antagonists potentiate the analgesic effects of morphine, endogenous enkephalins, and μ-receptor agonists (Watkins, Kinscheck, & Mayer, 1984; Katsuura & Itoh, 1985; Watkins, Kinscheck, Kaufman, Miller, Frenk, & Mayer, 1985; Dourish, O'Neill, Coughlan, Kitchener, Hawley, & Iversen, 1990; Dourish, O'Neill, Schaffer, Siegel, & Iversen, 1990; Zhou, Sun, Zhang, & Han, 1993; Valverde, Maldonado, Fournie-Zaluskie, & Roques, 1994; Vanderah, Bernstein, Yamamura, Hruby, & Porecca, 1996), enhance the ability of morphine to reduce c-Fos expression in the spinal cord (Chapman, Honore, Buritova, & Besson, 1995), and facilitate morphine induced inhibition of C-fiber activity (Kellstein, Price, & Mayer, 1991). Additionally, CCK antagonists impeed the acquisition of morphine tolerance (Tang, Chou, Iadarola, Yang, & Costa, 1984; Watkins et al., 1984; Dourish, Hawley, & Iversen, 1988; Dourish et al., 1990; Xu, Wiesenfeld-Hallin, Hughes, Horwell, & Hokfelt, 1992) yet have no effect on morphine dependence or withdrawal (Panerai,

Rovati, Cocco, Sacerdote, Mantegazza, 1987; Baber et al., 1989; Xu, et al., 1992; Maldonado, Valverde, Derrien, Tejedor-Real, & Roques, 1994). Although CCK antagonists potentiate the effects of opioids, they have not been shown to produce analgesia or alter nociceptive threshold levels when administered independently (Baber, et al., 1989; Vanderah, et al., 1996). Two distinct CCK receptors have been pharmacologically and electrophysiologically characterized in the rat and human CNS, the CCK-A receptor and the CCK-B receptor (Branchereau, Champagnat, Denavit-Saubie, 1993).

Investigators have suggested that a Pavlovian model of conditioned learning can be used to explain certain forms of drug tolerance (Tye, & Iversen, 1975; Siegel, 1975; Siegel, 1976; Siegel et al., 1981). For example, if an unconditional stimulus (UCS) such as morphine is repeatedly paired with specific environmental cues, then the environmental cues, acting as conditional stimuli (CS), will come to elicit a conditional response (CR), in this case, tolerance. As the number of morphine (UCS) and environmental (CS) pairings increases, morphine tolerance in the presence of the CS will be strengthened. As morphine tolerance is strengthened, less analgesia will be apparent following morphine administration (Siegel, 1976). Since the CR (tolerance) will act to counter the effects of the UCS (morphine), conditioned tolerance can be thought of as a compensatory response resulting from the anticipation of drug administration. Because the CR is only manifested in the presence of the CS, an animal tested in an environment which does not contain the CS will not display the CR (tolerance). While numerous studies

have demonstrated that such conditioned learning can indeed result from opioid administration, the biological mechanism by which this process occurs has yet to be identified.

In the present study we tested the hypothesis that CCK mediates associative (conditioned) tolerance, but is not involved in non-associative (non-conditioned) tolerance. CCK-A and CCK-B receptor antagonists were administered following the acquisition of either associative or non-associative tolerance and tail flick latency was measured to determine if a change in tolerance state had occurred.

Materials and Methods

Subjects

Male Sprague-Dawley rats (Charles River, Wilmington, Massachusetts) weighing 325-350 grams at the time of surgery were individually housed in hanging baskets from a Thoren rack. The colony room followed a standard 12 hour light-dark cycle with food and water available ad libitum. The temperature of the colony room was kept constant (21°C). Animals were tested at the same time during their light cycle each day.

Surgery

Prior to experimentation, animals were anesthetized with 50 mg/kg Nembutal, implanted with chronic jugular catheters (PE 50), and

allowed a one week recovery period. Group NA animals were anesthetized with halothane (1-4%) and administered 2 subcutaneous morphine pellets (75mg morphine base, NIDA) every 24 hours for 3 days following the recovery period.

Drugs

Morphine sulfate powder and morphine sulfate pellets (75mg) were obtained from the National Institute of Drug Abuse. L-365,260 and MK-329 were the generous gift of Merck Pharmaceuticals (UK). Morphine sulfate powder and MK-329 were dissolved in physiological saline. L-365,260 was disolved in 90% ETOH. Drug doses were determined based on published reports of the physiologically relevant and selective doses of these compounds for i.v. or s.c. administration (Dourish, Hawley, & Iversen, 1988; Higgins, Joharchi, Wang, Corrigall, & Sellers, 1994; Chapman, Honore, Buritova, & Besson, 1995; Rohde, Detweiler, & Basbaum, 1996).

Behavioral Apparatus

Tail flick latency was measured using a modified Hargreave's device. The Hargreave's device contained a radiant heat source which was situated beneath the glass floor of the experimental chamber and could be positioned beneath the tail of the animal. A photocell was affixed to the Hargreave's radiant heat source so that horizontal or vertical movement of the tail terminated the trial. A cut-off time of 12 seconds was utilized to avoid tissue damage. The behavioral

chambers measured 15x15x15 inches and consisted of either white or black plexiglas walls, a plexiglas ceiling, and a glass floor. i.v. injections were made using a 22 gauge needle and a 1.0cc syringe.

Behavioral Protocol

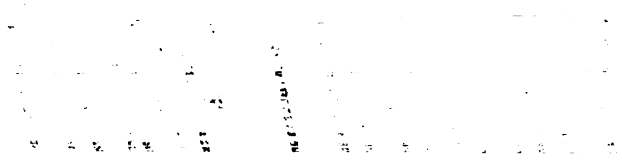
Animals were divided into two groups. Group A animals were administered morphine in an associative fashion, while Group NA animals were administered morphine in a non-associative fashion.

Group A (associative tolerance):

Rats were exposed to two distinct environments on alternating days for 16 days. Environments differed in terms of size, color, smell, tactile stimulation, and lighting. On the first day in each environment, rats were administered .05 cc physiological saline i.v. before being placed in the experimental chamber. Once inside the experimental chamber, animals were allowed a 15 minute habituation period. Tail flick latency was then measured every 10 minutes for 60 minutes for a total of 6 tail flick latency measurements per animal. Animals were then returned to their home cages. On consecutive days, animals were administered either 2.5 mg/kg morphine i.v. in a volume of .05cc physiological saline in the paired environment or .05cc physiological saline in the unpaired environment. Following i.v. administrations catheters were flushed with .2cc heparinized saline. Animals were allowed a 15 minute habituation period and then tail flick latency was measured as before. On experimental day 15 or 16 animals were administered

either .1 mg/kg MK-329 or .2 mg/kg L-365,260 i.v. in their home cages seven minutes prior to morphine administration. Following morphine administration animals were allowed to habituate to the experimental environment for 15 minutes. Tail flick latency was then measured as before. On experimental day 15 or 16 morphine was administered in the unpaired environment to confirm the development of associative tolerance. Experimental environments and testing days were counter-balanced.

Group NA (non-associative tolerance): On the first experimental day, animals were administered .05cc saline i.v. followed by a .2cc heparinized saline flush. Animals were then placed in one of two experimental environments and allowed a 15 minute habituation period. Tail flick latency was measured every 10 minutes for 60 minutes for a total of 6 tail flick latency measurements per animal. Animals were then returned to their home cages. Sixty minutes later, animals were anesthetized with halothane and administered 2 subcutaneous morphine pellets. Following the procedure, animals were returned to their home cages. On the second and third experimental days, animals were placed in one of the two experimental environments and allowed a 75 minute habituation period. Animals were then returned to their home cages for 60 minutes, after which animals were anesthetized and administered 2 subcutaneous morphine pellets as before. On the last experimental day, animals were divided into 2 groups. Group 1 received either .1 mg/kg MK-329 or .2 mg/kg L-365,260 seven minutes prior to the i.v. administration of 2.5 mg/kg morphine. Group 2 received either .1



mg/kg MK-329 or .2 mg/kg L-365,260 45 minutes following the i.v. administration of 2.5 mg/kg morphine. Following morphine administration, group 1 animals were placed in one of the experimental environments and allowed a 15 minute habituation period. Tail flick latency was measured every 10 minutes for 60 minutes for a total of 6 tail flick latency measurements. Following morphine administration, group 2 animals were placed in one of the experimental environments and allowed a 15 minute habituation period before tail flick latency was measured every 5 minutes for 30 minutes for a total of 6 tail flick latency measurements. Group 2 animals were then removed from the experimental environment, administered either MK-329 or L-365,260 and then placed back in the experimental environment. Animals were allowed a 15 minute habituation period. Tail flick latency was measured every 5 minutes for 30 minutes for a total of 6 tail flick latency measurements per animal. Care was taken to treat NA animals in such a way as to prevent the association of morphine administration with any environmental cues.

Note: a preliminary group of animals (n=8) were administered s.c. morphine pellets every day for 5 days, after which time tail flick latencies were no longer significantly different from baseline values. On day 6, these animals were administered .2mg/kg L-365,260 seven minutes prior to receiving a challenge dose of morphine (2.5 mg/kg, i.v.). Tail flick latency was not significantly altered following the administration of the challenge dose. Since the animals were completely tolerant at the time of testing, it seemed possible that any

anti-tolerant effect of L-365,260 could have been obscured by the magnitude of morphine tolerance. In order to increase the sensitivity of our methods, subsequent animals were pelleted for only three days such that tolerance was only partial and the effects of L-365,260 administration could be more clearly observed.

Statistical Analysis

The mean of the 6 tail flick latencies measured for each animal was calculated for each test day. As the data did not follow a standard distribution (due to the use of a 12 second cut-off) non-parametric statistics were employed for data analysis. Wilcoxon two group signed rank tests were used to compare the mean tail flick latencies on the different test days. Statistical significance was set at $p<.01$. All statistical tests were conducted using GB-STAT, version 5.1.2. All figures were created using Stat View, version 4.0, and Adobe Illustrator, version 5.5.

Results

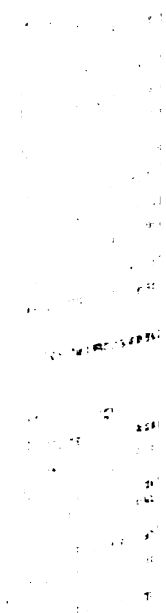
Comparison of tail flick latency measurements taken on the last four experimental days indicate that while Group A rats had become tolerant to morphine in the paired environment, they did not exhibit tolerance to morphine in the unpaired environment (Figure 1). Thus, the morphine tolerance observed under these conditions was completely associative in nature. Group NA animals were tolerant to morphine in all experimental environments (data not shown)

indicating that the morphine tolerance observed in these animals was non-associative.

Following the systemic administration of the CCK-B antagonist L-365,260, associative tolerance was completely reversed (Figure 2). However, systemic administration of the CCK-A antagonist MK-329 did not affect associative tolerance (Figure 2). Neither L-365,260 nor MK-329 had an effect on non-associative tolerance when administered either prior to or following the administration of morphine (Figure 3).

Discussion

Our data indicate that when animals are exposed to an environment which has been repeatedly paired with the administration of morphine, a CCK-mediated compensatory response develops and serves to antagonize the effects of the morphine. The data also suggest that CCK is acting at CCK-B receptors to produce this effect. With this evidence, several predictions can be made. First, if conditioned tolerance contributes to the gradual decrease in analgesic efficacy of clinically prescribed opioids then the co-administration of a CCK-B antagonist should prevent or reverse this effect. Second, researchers have noted that incidents of overdose are more likely in heroin-tolerant rats when they are administered heroin in a novel environment (Siegel, et al., 1982). Similarly, accidental overdose occurs more frequently in human heroin addicts following drug administration in a novel or unusual environment (Siegel, 1984). It



is possible that when environmental cues signaling impending opioid administration are not apparent a CCK-mediated compensatory response is not triggered. In such cases, the administration of a CCK-B antagonist should prevent the conditioned tolerance from developing and therefore preclude overdose.

Conclusions

In summary, we have demonstrated that the CCK-B receptor antagonist L-365,260 reverses associative but not non-associative tolerance. In contrast, the CCK-A receptor antagonist MK-329 has no effect on either associative or non-associative tolerance. Since there may be differences in the distribution and function of CCK receptors between the rodent and primate, further studies will be necessary to determine the role of CCK in conditioned opioid tolerance in human subjects and to identify the brain regions involved in this effect. Our findings suggest that CCK mediates a learned, environmentally specific, compensatory mechanism which produces associative opioid tolerance, and that CCK-B antagonists may be important in the treatment of opioid tolerance and addiction.

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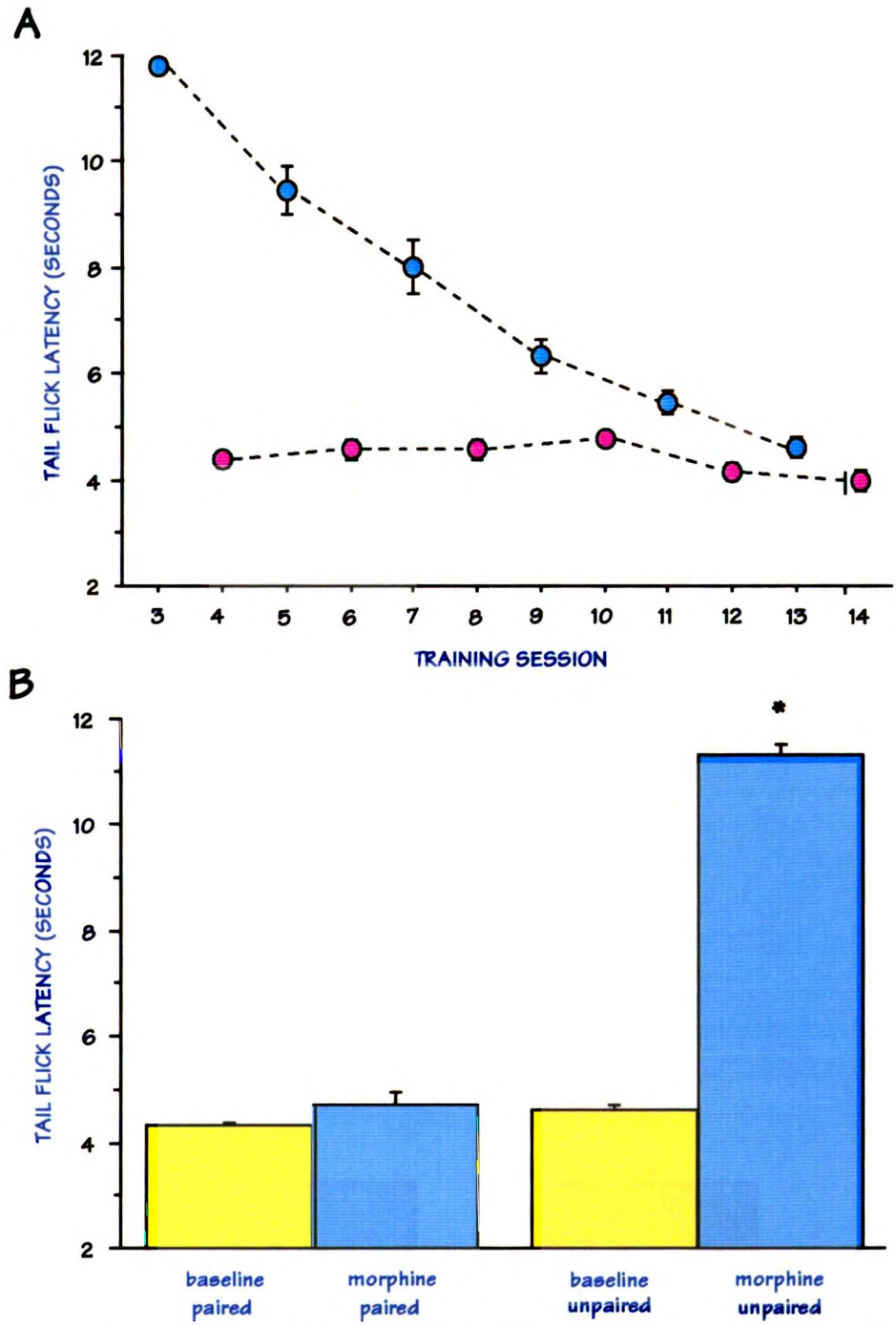

Figure 1: A) Demonstration of the acquisition of associative tolerance (n=24). = i.v. administration of saline in the unpaired environment on even numbered days. = i.v. administration of morphine (2.5mg/kg) in the paired environment on odd numbered days. B) Associative tolerance is context dependent. Tail flick latency (n=19) in paired and unpaired environments before ( ) and after ( ) the acquisition of associative tolerance to morphine (2.5 mg/kg) in the paired environment. Baseline tail flick latencies were measured on experimental days 1 and 2, respectively. Morphine paired tail flick latencies were measured on experimental day 13. Morphine unpaired tail flick latencies were measured on either experimental day 15 or 16. Animals were tolerant to the morphine administered in the paired environment by the 6th administration (day 13) but were not tolerant to the same dose of morphine given in the unpaired environment ($p<.01$). There was no significant difference in tail flick latency between the baseline and morphine conditions in the paired environment following the acquisition of associative tolerance. Tail flick latency was significantly different between the baseline and morphine conditions in the unpaired environment following the acquisition of associative tolerance ($p<.01$). Error bars represent standard error of the means.

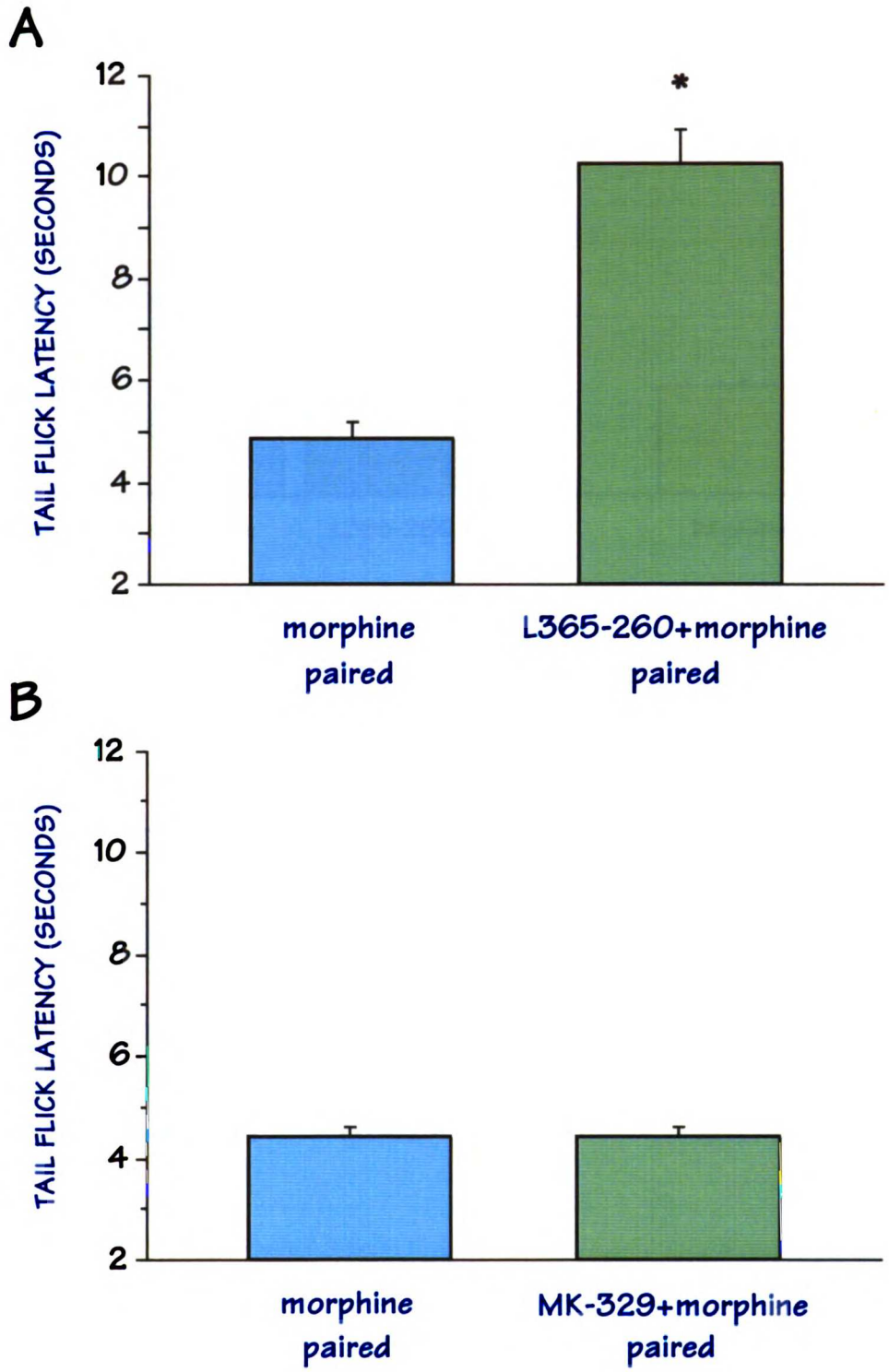

Figure 2: A) Associative tolerance is reversed by the systemic administration of the CCK-B antagonist L365-260 (.2 mg/kg, n=11, p<.01). B) Associative tolerance is not reversed by the systemic administration of theCCK-A antagonist MK-329 (.1 mg/kg, n=13). Morphine was administered in the paired environment on experimental day 13. L365-260 and MK-329 were administered 7 minutes prior to systemic morphine administration on experimental day 15 or 16. Error bars represent standard error of the means.

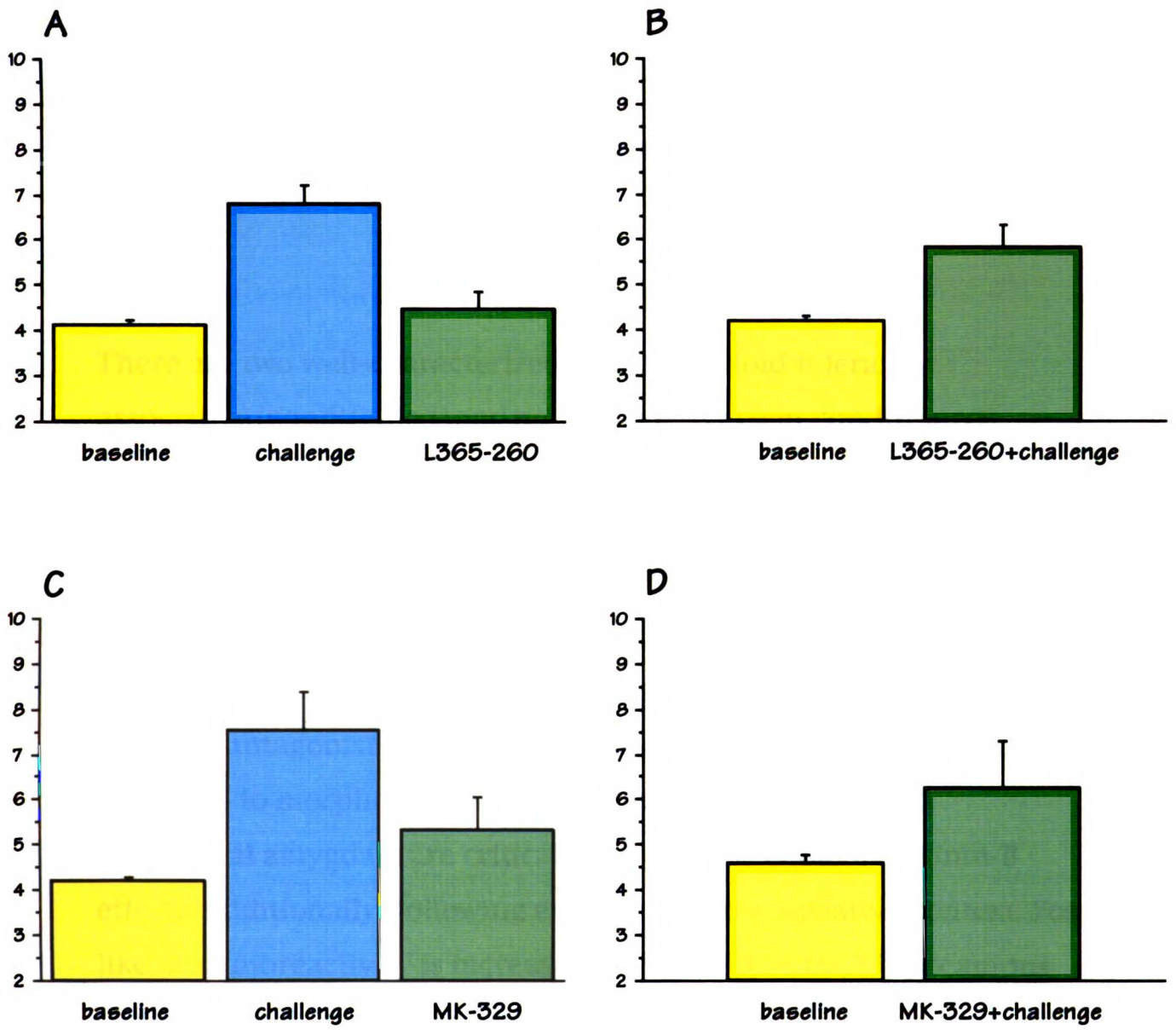

Figure 3: Lack of reversal of non-associative tolerance following the systemic administration of L365-260 or MK-329. A) L365-260 was administered 45 minutes after the administration of a challenge dose of morphine (2.5 mg/kg, i.v., n=6). B) L365-260 was administered 7 minutes prior to systemic morphine administration (2.5 mg/kg, i.v., n=7). C) MK-329 was administered 45 minutes after the administration of a challenge dose of morphine (2.5 mg/kg, i.v.,n=6). D) MK-329 was administered 7 minutes prior to systemic morphine administration (2.5 mg/kg, i.v., n=7). There were no significant differences when $p<.01$. Error bars represent standard error of the means.

Chapter 5:

A locus and neural mechanism for associative morphine tolerance

Abstract

There are two well-characterized forms of opioid tolerance: associative (conditioned) and non-associative (non-conditioned). Associative tolerance results from the repeated administration of an opioid in the presence of specific environmental cues while non-associative tolerance results from the repeated administration of an opioid without consistent contextual pairing. We have previously demonstrated that a systemically administered cholecystokinin-B receptor antagonist reverses associative, but not non-associative, tolerance to morphine. Here we report that the lateral and basolateral amygdala are critical targets of the cholecystokinin-B effect. Additionally, following exposure to an associative context, Fos like immunoreactivity is increased in area CA1 of the hippocampus as well as in the lateral and basolateral amygdala. These results support the hypothesis that the hippocampus and amygdala are involved in associative morphine tolerance and elucidate a novel circuit of potential importance for the treatment of opioid tolerance andaddiction.

Introduction

There is considerable evidence that cholecystokinin (CCK) can counteract the analgesic effects of opioids. Systemically

administered CCK attenuates or prevents morphine induced analgesia and some CCK antagonists potentiate the analgesic effects of opioids and impede the development of morphine tolerance (Faris, Komisaruk, Watkins, & Mayer, 1983; Watkins, Kinscheck, & Mayer, 1984; Watkins, Kinscheck, Kaufman, Miller, Frenk, & Mayer, 1985; Baber, Dourish, & Hill, 1989; Dourish, O'Neill, Coughlan, Kitchener, Hawley, & Iversen, 1990; Valverde, Maldonado, Fournie-Zaluski, & Roques, 1994). Additionally, our laboratory recently demonstrated that a systemically administered CCK antagonist reverses associative, but not non-associative, morphine tolerance and that this involves an action at the CCK-B receptor. In the present study, we attempted to determine the CNS sites of action of CCK's systemic effects on associative tolerance using Fos immunohistochemistry and the microinjection of the CCK-B receptor antagonist L-365,260.

Conditioned learning can contribute to drug tolerance and overdose in rodents and humans (O'Brien, O'Brien, Mintz, & Brady, 1975; Siegel, 1976; Siegel, Hinson, & Krank, 1981; Siegel, Hinson, Krank, & McCully, 1982; Siegel, 1984; O'Brien, Ehrman, & Ternes, 1986; O'Brien, Childress, Ehrman, & Robbins, 1998). When drug administration is paired with specific environmental cues then these cues can act as conditioned stimuli and can elicit a conditioned response, such as tolerance. Researchers have suggested that this type of learned tolerance may be serving as a compensatory response; acting to maintain a state of homeostasis within the organism. Typically, such homeostasis is the result of negative feedback. For example, opioid administration can lead to analgesia.

With repeated administrations, an animal may compensate for the analgesia through a mechanism that produces hyperalgesia when the opioid is presented. Eventually, this hyperalgesia can completely block the analgesia produced by the drug. The result will be that opioid administration no longer produces analgesia (i.e. tolerance). The mu opioid receptor is necessary and sufficient for morphine analgesia (Matthes, Maldonado, Simonin, Valverde, Slowe, Kitchen, Befort, Dierich, Le Meur, Dolle, Tzavara, Honoune, Roques, & Kieffer, 1996). In contrast to non-associative (non-conditioned) morphine tolerance, associative (conditioned) morphine tolerance is not specific to the mu receptor (Carter & Tiffany, 1996). This suggests that these two types of tolerance involve different CNS circuitry. This hypothesis was addressed in the present study. Once we established that regions of the hippocampus and amygdala were involved in associative tolerance, we hypothesized that the previously observed systemic effects of the CCK-B receptor antagonist L-365,260 on associative tolerance would be replicated by microinjecting this compound into one of these brain regions.

Materials and Methods

Subjects

Male Sprague-Dawley rats (n=64, Charles River, Wilmington, Massachusetts) weighing 300-325 grams at the time of surgery were individually housed in hanging baskets from a Thoren rack. The colony room followed a standard 12 hour light-dark cycle with food

and water available ad libitum. The temperature of the colony room was kept constant (21°C). Animals were tested at the same time during their light cycle each day.

Surgery

Prior to experimentation, animals were anesthetized with 50 mg/kg Nembutal, implanted with chronic jugular catheters (PE 50), and allowed a one week recovery period. Group A animals in the microinjection study were implanted bilaterally with stainless steel 26 gauge chronic guide cannulae (Plastics One, Roanoke, Virginia). Group NA animals were anesthetized with halothane (1-4%) and administered 2 subcutaneous morphine pellets (75mg morphine base, NIDA) every 24 hours for 4 days following the recovery period.

Drugs

Morphine sulfate powder was obtained from the pharmacy at the University of California, San Francisco, and was dissolved in physiological saline for i.v. microinjections. Subcutaneous morphine pellets (75 mg morphine base) were obtained from the National Institute of Drug Abuse (NIDA). The CCK-B antagonist L-365,260 was the generous gift of Dr. Les Iversen and was dissolved in 90% ETOH for i.c. microinjections. Drug doses were determined based on published reports of the physiologically relevant and selective doses of these compounds following i.v., s.c., or i.c. microinjections (Crawley, 1992; Rohde, Detweiler, & Basbaum, 1996).

Behavioral Apparatus

Tail flick latency was measured using a modified Hargreave's device (Mitchell, Lowe, & Fields, 1998). The Hargreave's device contained a radiant heat source which was situated beneath the glass floor of the experimental chamber and could be positioned beneath the tail of the animal. A photocell was affixed to the Hargreave's radiant heat source so that horizontal or vertical movement of the tail terminated the trial. A cut-off time of 12 seconds was utilized to avoid tissue damage. The behavioral chambers measured 15x15x15 inches and consisted of either white or black plexiglas walls, a plexiglas ceiling, and a glass floor. i.v. injections were made using a 22 gauge needle and a 1.0cc syringe.

Behavioral Protocol

Animals were divided into two groups: associative (A) and non-associative (NA). Group A animals were administered morphine in an associative fashion, while Group NA animals were administered morphine in a non-associative fashion.

<u>Group A (associative tolerance):</u>
Rats were exposed to two distinct environments on alternating days for 16 days. Environments differed in terms of size, color, smell, tactile stimulation, and lighting. On the first day in each environment, rats were administered 0.05 cc physiological saline i.v.

before being placed in the experimental chamber. Once inside the experimental chamber, animals were allowed a 15 minute habituation period. Tail flick latency was then measured every 10 minutes for 60 minutes for a total of 6 tail flick latency measurements per animal. Animals were then returned to their home cages. On consecutive days, animals were administered either 2.5 mg/kg morphine i.v. in a volume of 0.05cc physiological saline in the morphine-paired environment or 0.05cc physiological saline in the saline-paired environment. Following i.v. administrations catheters were flushed with 0.2cc heparinized saline. Animals were allowed a 15 minute habituation period and then tail flick latency was measured as before. For Fos Study: On the last experimental day (day 15), Group A animals were divided into two groups. Half of the Group A animals (n=4) received saline in the saline context (AS) and half (n=4) received morphine in the morphine context (AM). 90 minutes after drug administration on the last experimental day, all animals were perfused, their brains removed, and processed for Fos. For Microinjection Study: On experimental day 15 or 16 animals were administered L-365,260 i.c. (15ng in 0.5µl per brain site) in their home cages seven minutes prior to morphine administration. Following morphine administration, animals were allowed to habituate to the experimental environment for 15 minutes. Tail flick latency was then measured as before. On experimental day 15 or 16 morphine was administered in the saline-paired environment to confirm that the tolerance that developed was associative. Experimental environments and testing days were counter-balanced. Thus, animals that received L-365,260 on day 15 received morphine

in the saline-paired environment on day 16, and vice verse. Animals that received microinjections into the L/BL amygdala participated in one additional experimental day. On day 17, these animals were divided randomly into two groups. Half of the animals were microinjected with L-365,260 (15ng in 0.5µl per brain site) in the saline-paired environment while the other half received volume-matched microinjections of vehicle (90% ETOH) in the saline paired environment. Tail flick latency was then measured as before.

Group NA (non-associative tolerance): On the first experimental day, animals were administered 0.05cc saline i.v. followed by a 0.2cc heparinized saline flush. Animals were then placed in one of two experimental environments and allowed a 15 minute habituation period. Tail flick latency was measured every 10 minutes for 60 minutes for a total of 6 tail flick latency measurements per animal. Animals were then returned to their home cages. Sixty minutes later, animals were implanted with 2 subcutaneous morphine pellets and then returned to their home cages. On days 2-5, animals were placed in one of the two experimental environments and allowed a 75 minute habituation period. Animals were then returned to their home cages for 60 minutes, after which animals were anesthetized and administered 2 subcutaneous morphine pellets as before. On the last experimental day (day 6), Group NA animals were divided into two groups. Half (n=4) received saline in the saline context (NAS) and half (n=4) received morphine in the morphine context (NAM). 90 minutes after drug administration on the last experimental day, all animals were perfused, their brains removed, and processed for

Fos. Care was taken to prevent the association of morphine administration with any environmental cues.

Microinjections

All injection sites were determined based on the atlas of Paxinos & Watson (1986). Bilateral microinjections were made into the following brain structures: VTA (n=7, AP: -5.8, ML: +/-.5, DV: -8.5), NAcc core (n=10, AP: 2.5, ML: +/-1.2, DV: -6.5), CA1 (n=12, AP: -3.6, ML: +/-1.5, DV: -3.0), CeA (n=7, AP: -2.5, ML: +/-4.3, DV: -8.3), L/BLA (n=12, AP: -2.8, ML: +/-5.2, DV: -8.5). Injections were made using a 1µl Hamilton Syringe (Hamilton Co., Reno, Nevada) attached to a 10 cm length of PE 50 tubing which, in turn, was connected to a 33 gauge injection cannula (Plastics One). Microinjections were conducted at a rate of 0.5µl per minute. Injection cannula extended 2mm past the guide cannula and were left in place for one minute following microinjection to minimize the flow of drug solution up the cannula track.

Immunohistochemistry

Animals were perfused with 150 ml 0.1M phosphate buffered saline followed by 400 ml of 4% formaldehyde in 0.1M phosphate buffer. Following the perfusion, brains were removed and post-fixed for 2 hours at room temperature. Tissue was then transferred to a 30% sucrose solution and left overnight prior to cutting on a sliding microtome. Tissue was cut in 50µm sections and washed in a

solution of 0.05M tris-phosphate buffered saline with 1% normal goat serum and 0.3% Triton-X prior to being incubated for one hour at room temperature in a blocking solution of .05M tris-phosphate buffered saline with 3% normal goat serum and 0.3% Triton-X. Primary antibody (rabbit polyclonal, generously provided by Dr. Dennis Slamon) was then applied to the tissue. Tissue was incubated overnight at room temperature. Sections were thoroughly washed prior to the application of secondary antibody (goat anti-rabbit IgG and avidin-biotin-peroxidase complex, Vector Labs, Burlingame, California). Secondary antibody was left on the tissue for a period of 2 hours. A nickel diaminobenzidine (DAB) reaction (Llewellyn-Smith et al.) was employed to visualize Fos immunoreactivity. Brain tissue was processed simultaneously for Group A and Group NA animals to control for variability in, and minimize artifact from, the reagents used.

FosQuantification

Fos IR positive cells were counted in each of the following brain regions; nucleus accumbens core and shell, ventral tegmental area, area CA1 of the hippocampus, central nucleus of the amygdala, basolateral nucleus of the amygdala, and lateral nucleus of the amygdala. These regions were selected based on their demonstrated involvement in learning, reinforcement, and opioid analgesia.

Sections were examined under dark-field illumination to identify the topographical boundaries of each brain region of interest. To

simplify the quantification process, regional boundaries were defined as follows: nucleus accumbens (NAcc): AP 2.2 to AP .70, ventral tegmental area (VTA): AP -5.2 to AP -6.8, area CA1 of the hippocampus (CA1): AP -2.3 to AP -4.30, central nucleus of the amygdala (CeA): AP -1.8 to AP -3.0, basolateral nucleus of the amygdala (BLA): AP -1.8 to AP -3.3, & lateral amygdala (LA): AP -2.5 to AP -3.3. This rendered a total of 30 sections per animal for the NAcc, 32 sections per animal for the VTA, 40 sections per animal for CA1, 24 sections per animal for the CeA, 30 sections per animal for the BLA, and 16 sections per animal for the LA. Fos positive cells were quantified at 20x magnification using light-field illumination. Fos positive cells were counted by an observer blind to the experimental treatment of each animal. Once quantification was completed, the total number of cells for each animal in each brain region was divided by the number of sections per region, rendering a mean number of Fos cells for each animal for each brain nucleus.

Statistical Analysis

For Fos Study: A one-way randomized ANOVA was used to analyze the data collected from each brain region. Tukey-Kramer post-hoc tests were conducted for pairwise comparisons. For Microinjection Study: The mean of the 6 tail flick latencies measured for each animal was calculated for each test day. As the data did not follow a standard distribution (due to the use of a 12 second cut-off) non-parametric statistics were employed for data analysis. Wilcoxon two group signed rank tests were used to compare the mean tail flick

latencies on the different test days. Statistical significance was set at $p<.05$. All statistical tests were conducted using GB-STAT, version 6.5. All figures were created using Stat View, version 4.0, and Adobe Illustrator, version 5.5.

Results

No significant differences were found between the four groups (AS, AM, NAS, & NAM) in the VTA, NAcc core, NAcc shell, and Ce amygdala (Figure 1). In contrast, there was a significant main effect of group in CA1 ($p=.0002$), L amygdala ($p=.0004$), and BL amygdala ($p=.0193$) (Figure 1). Post-hoc tests revealed that in CA1, the AM group had significantly more Fos positive cells than the NAM and NAS groups ($p<.01$). Additionally, the AS group had significantly more Fos positive cells than the NAM and NAS groups ($p<.05$). In L amygdala, post-hoc tests revealed that the AM group had significantly more Fos positive cells than all other groups ($p<.01$). Similarly, in the BL amygdala, the AM group had a significantly greater number of Fos positive cells than the NAM and NAS groups ($p<.05$). These results indicate that CA1, L amygdala, and BL amygdala are active when an animal is exposed to a context associated with morphine administration as compared to one associated with saline administration.

To determine if the reversal of associative tolerance following the systemic administration of the CCK-B antagonist L-365,260 was due to an action in either the amygdala or hippocampus, L-365,260 was

microinjected into CA1 and L/BL amygdala following the acquisition of associative tolerance. Injections were also made into Ce amygdala, VTA, and NAcc core because these nuclei also express the CCK-B receptor (Honda, Wada, Battey, & Wank, 1993; Shigeyoshi, Okamura, Inatomi, Matsui, Ito, Kaji, Abe, Nakata, Chiba, & Chihara, 1994; Crawley & Corwin, 1994; Mercer, Beart, Horne, Finkelstein, Carrive, & Paxinos, 1996; Reum, Schafer, Marsden, Fink, & Morgenstern, 1997).

As illustrated in Figure 2, there was a significant main effect for group ($p<.0001$). Post-hoc tests revealed that a significant change in associative morphine tolerance was not apparent following the microinjection of L-365,260 into VTA, NAcc core, and CA1. However, the microinjection of L-365,260 into Ce amygdala and L/BL amygdala did result in a significant attenuation of associative morphine tolerance ($p<.01$ for both nuclei). The effect of L-365,260 in L/BL amygdala was not significantly different from that in Ce amygdala. Furthermore, it is important that this effect was also not significantly different from the systemic effect observed in our previous study (Figure 3). In contrast, the effect of L-365,260 in Ce amygdala was significantly different from the previously observed systemic effect of L-365,260 ($p<.05$). This suggests that CCK in L/BL amygdala is required for the expression of associative morphine tolerance and indicates that a CCK action in L/BL amygdala is critical to the attenuation of associative morphine tolerance previously observed following the systemic administration of L-365,260. Given the proximity of the L/BL amygdala to the Ce amygdala and the data

from our Fos study, it seems most likely that the effect of L-365,260 in Ce amygdala is due to diffusion of the drug into L/BL amygdala. Taken together, we suggest that neurons in the L/BL amygdala are activated by exposure to the morphine-associated context and that CCK acting in this brain region is necessary for the expression of associative morphine tolerance.

To ensure that the effects observed after the microinjection of L-365,260 into L/BL amygdala were not due to the vehicle or injection volume used nor to a non-specific effect of L-365,260, control injections of vehicle or L-365,260 were made into L/BL amygdala in the saline-paired environment (Figure 4). ANOVA analysis revealed a significant main effect for group. Post-hoc tests demonstrated that, following the acquisition of associative tolerance, while the administration of L-365,260 in the morphine-paired environment significantly attenuated morphine tolerance, injections of vehicle or L-365,260 in the unpaired environment had no effect. This indicates that the effect of L-365,260 in the L/BL amygdala is restricted to the morphine-paired environment and is due to an action at the CCK-B receptor.

Discussion

The contribution of the hippocampus to learning and memory is clearly established (Squire, 1992; Zola-Morgan & Squire, 1993; Alvarez, Zola-Morgan, & Squire, 1995; Morris & Frey, 1997; Reed & Squire, 1997). Furthermore, it has been suggested that CCK is

involved in the acquisition and retrieval of emotionally based memory through an action in the hippocampus (Lemaire, Bohme, Piot, Roques, & Blanchard, 1994). Although our investigation did not establish a contribution of the hippocampus via a CCK-B based mechanism, the Fos analysis suggests that CA1 contributes to either the acquisition or expression of associative morphine tolerance. There is evidence that the hippocampus serves as a locus for memory consolidation and performs the match-mismatch discriminations necessary for memory recognition (Eichenbaum, Otto, & Cohen, 1994; Young, Otto, Fox, & Eichenbaum, 1997). Moreover, there are direct, reciprocal connections between the hippocampus and L/BL amygdala (Amaral, 1986; Saunders & Rosene, 1988). Therefore, given the current findings, we suggest that the projection from CA1 to the L/BL amygdala contributes to the formation of associative tolerance, and that the acquisition or expression of associative tolerance would not occur in the absence of CA1. Further studies are needed to test these possibilities.

Our results indicate that CA1 is active following exposure to either the saline-paired or morphine-paired context. This suggests that the saline-paired context is no less salient to the animal than the morphine-paired context. When an animal is exposed to an associative environment it must process the available sensory information in order to determine whether the environment signals impending drug administration. In the present study, both contexts contain relevant information regarding drug delivery and both involve similar sensory processing. Therefore, it is possible that the

environmental discriminations that take place in the morphine-paired and saline-paired environments involve similar mechanisms in CA1 of the hippocampus.

The amygdala has been implicated in the encoding of affective memories (Le Doux, 1986; LeDoux, Iwata, Cicchetti, & Reis, 1988; McGaugh & Cahill, 1997; Aggleton & Mishkin, 1986) and in the expression of stimulus-reward associations (Jones & Mishkin, 1972; Spiegler & Mishkin, 1981). Additionally, neurons in the amygdala change their pattern of firing in response to emotionally significant stimuli (Rolls, 1981; 1986). Taken together with our current results, these data suggest that the L/BL amygdala is necessary for the expression of the association between particular environmental stimuli and drug delivery. We predict that in the absence of this brain region the appropriate pairing between stimulus and reward cannot occur and therefore associative tolerance would not be manifested.

The amygdala is sensitive to the effects of opioid and anti-opioid peptides. When injected into the amygdala, the opioid antagonists naloxone and naltrexone enhance memory retention (Introini-Collison, Nagahara, & McGaugh, 1989) and the intra-amygdaloid administration of CCK agonists facilitate learning and memory (Belcheva, Belcheva, Petkov, & Petkov, 1994; Huston, Schildein, Gerhardt, Privou, Fink, & Hasenohrl, 1998). Also, because CCK mRNA expression increases in the amygdala following repeated morphine administration, it has been suggested that the amygdala is the site of

a CCK mediated compensatory mechanism that contributes to opioid tolerance (Pu, Zhuang, Lu, Wu, & Han, 1994). Our results further indicate that the contextual cues that signal impending drug administration are required to evoke the release of CCK in the L/BL amygdala and that this is necessary for the expression of associative tolerance. We postulate that CCK release in the amygdala is part of a contextually triggered compensatory action that serves to maintain homeostasis. Furthermore, we suggest that this compensatory action does not occur in non-associative tolerance because the required relationship between contextual sensory stimuli and drug administration is not present.

Recently, investigators have suggested that neurons in the hippocampus and amygdala are part of a mechanism of learning and memory which is necessary for processing and assigning value to drug associated cues and which contributes to drug addiction (White, 1996). Our results further indicate that the hippocampus and amygdala act together to create an association between a specific environment and the rewarding effects of morphine administration. We hypothesize that during the acquisition phase of associative tolerance, CA1 is important for the match-mismatch discrimination that occurs when an animal is exposed to an associative environment and that the L/BL amygdala is important for assigning affective value and motivational state to the associative environment. Therefore, we predict that the acquisition of associative tolerance will be impaired when hippocampal connections are disrupted.

Drug addicts experience a compensatory or anticipatory response when exposed to environmental stimuli associated with drug administration (Ehrman, Ternes, O'Brien, & McLellan, 1992) and both humans and animals experience context-specific withdrawal when re-exposed to an environment previously paired with morphine (O'Brien, et al., 1975; O'Brien et al., 1986; Falls & Kelsey, 1989). Moreover, both animals and human addicts are more likely to overdose in novel environments not associated with previous drug intake (Siegel et al., 1982; Siegel, 1984). This observation has been proposed to result from the failure to trigger a compensatory mechanism in the absence of the environmental cues that typically precede drug administration. If CCK is responsible for such a compensatory mechanism then the present results predict that it might be possible to effectively reverse a heroin or morphine overdose by administering a CCK-B agonist.

Recent research has implicated the amygdala in drug-related craving. In human subjects, PET studies have shown that μ-opioid receptor binding (Zubieta, Gorelick, Stauffer, Ravert, Dannals, & Frost 1996) and glucose metabolism (Grant, London, Newlin, Villemagne, Liu, Contoreggi, Phillips, Kimes, & Margolin,1996) in the amygdala are correlated with subjective reports of cocaine craving, as is fMRI activation (Breiter, Gollub, Weiskoff, Kennedy, Makris, Berke, Goodman, Kantor, Gastfriend, Riorden, Mathew, Rosen, & Hyman, 1997). Additionally, it has been demonstrated that craving and drug seeking behavior are correlated with DA release in the amygdala (Tran-Nguyen, Fuchs, Coffey, Baker, O'Dell, & Neisewander, 1998).

However, the relationship between drug administration, conditioned tolerance, and craving is currently unclear. If, as numerous clinical studies suggest (O'Brien et al., 1986; Grant et al., 1996; Maas, Lukas, Kaufman, Weiss, Daniels, Rogers, Kukes, & Renshaw, 1998), an addict experiences a state of craving when exposed to objects or an environment previously paired with drug administration, then conditioned tolerance and craving may involve similar or overlapping mechanisms. If this is the case, then a drug which attenuates conditioned tolerance may have some effects on drug craving. Thus, it might also be possible to administer a CCK-B antagonist to an opioid addict in an environment previously associated with drug administration and attenuate not only the associative tolerance but some of the symptoms of craving. These predictions could have important implications for the treatment of human addicts.

Conclusions

In conclusion, our findings indicate that non-associative and associative tolerance involve different patterns of Fos activity in CA1 of the hippocampus and the L/BL amygdala. Specifically, we have demonstrated that limbic areas which have enhanced Fos expression under conditions of associative tolerance do not show these changes under conditions of non-associative tolerance. Additionally, we have demonstrated that the learned compensatory mechanism that contributes to associative morphine tolerance involves CCK acting at the CCK-B receptor in L/BL amygdala. Further research is necessary

to ascertain the origin of the endogenous CCK, to determine the contribution of the hippocampus to the formation and expression of associative morphine tolerance, and to fully elucidate the mechanism of action of the amygdaloid-mediated compensatory response. Our results also suggest that L/BL amygdala is an important target locus for the development of treatments for certain drugs of abuse.

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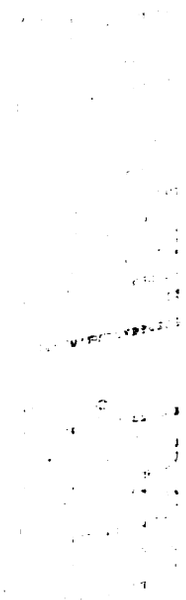

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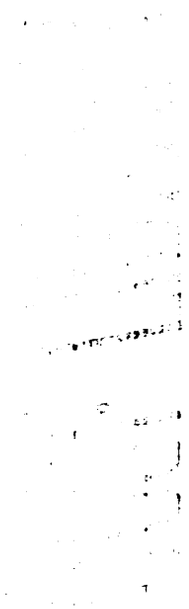

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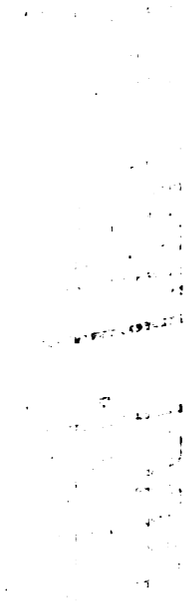

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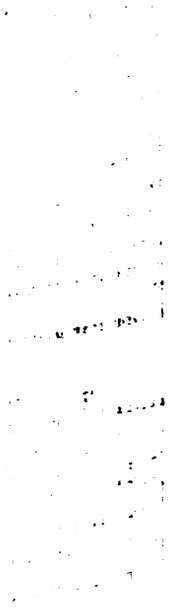

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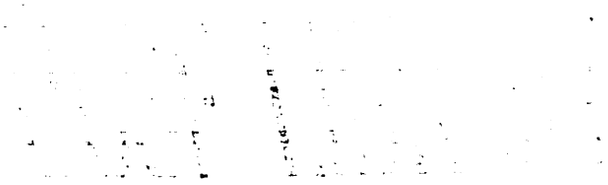


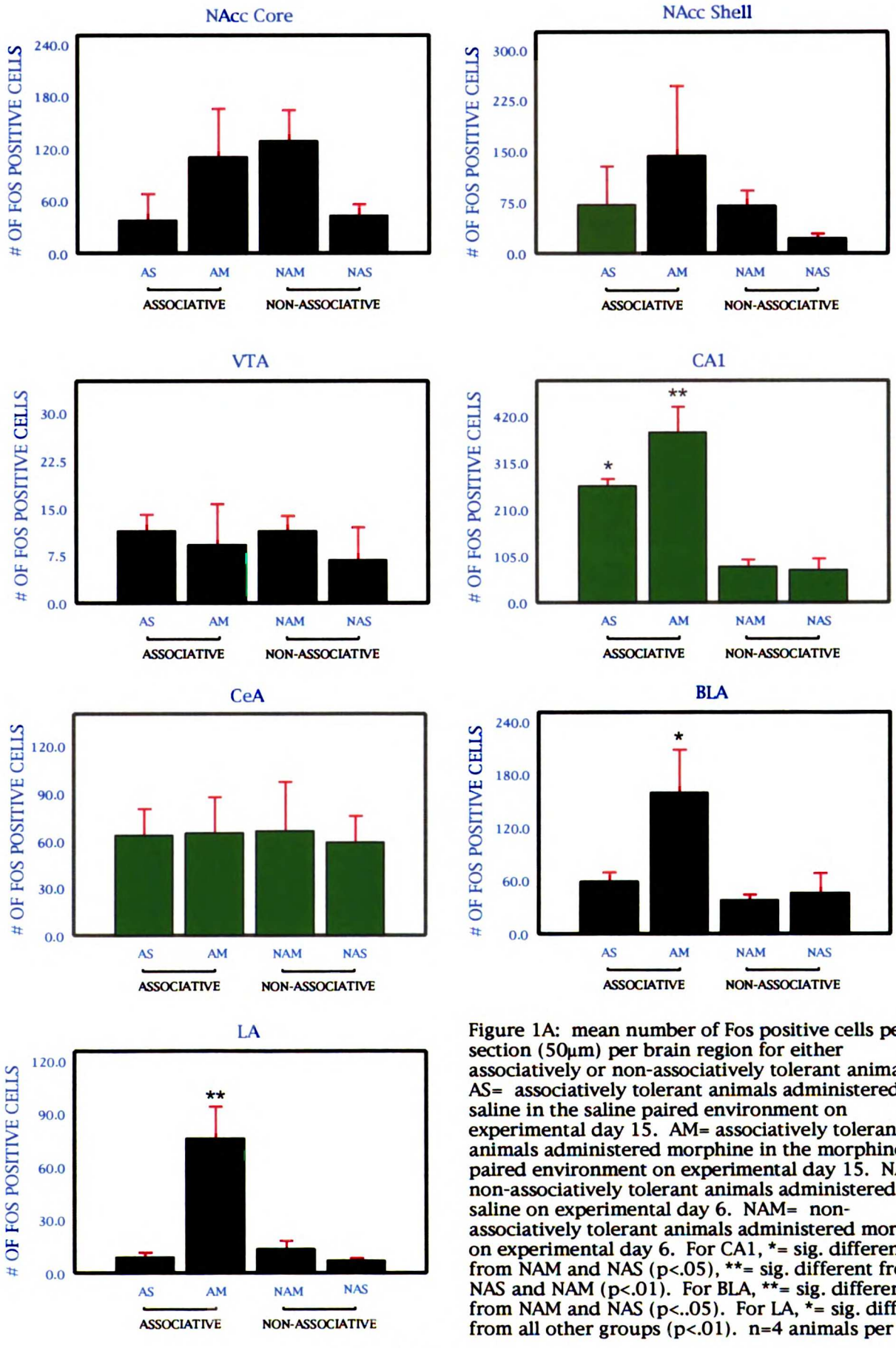


Figure 1A: mean number of Fos positive cells per section (50μm) per brain region for either associatively or non-associatively tolerant animals. AS= associatively tolerant animals administered saline in the saline paired environment on experimental day 15. AM= associatively tolerant animals administered morphine in the morphine paired environment on experimental day 15. NAS= non-associatively tolerant animals administered saline on experimental day 6. NAM= non-associatively tolerant animals administered morphine on experimental day 6. For CA1, *= sig. different from NAM and NAS ($p<.05$), **= sig. different from NAS and NAM ($p<.01$). For BLA, **= sig. different from NAM and NAS ($p<..05$). For LA, *= sig. different from all other groups ($p<.01$). n=4 animals per group.

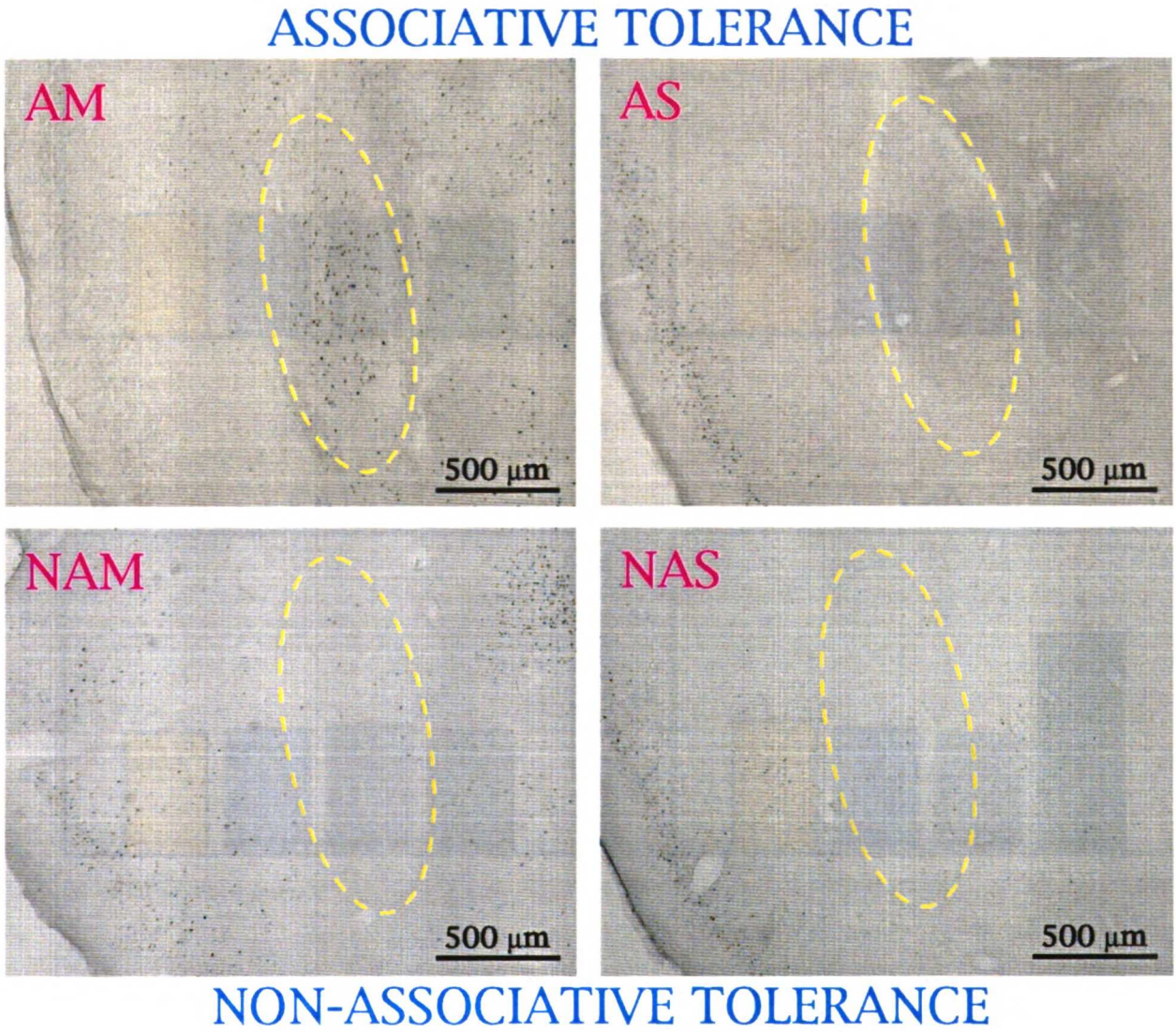
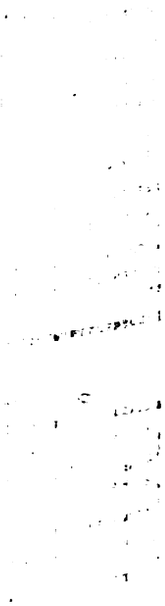


Figure 1B: Photomicrographs of Fos positive cells in the amygdala of associatively and non-associatively tolerant animals. AS= associatively tolerant animals administered saline in the saline-paired environment on experimental day 15. AM= associatively tolerant animals administered morphine in the morphine-paired environment on experimental day 15. NAS= non-associatively tolerant animals administered saline on experimental day 6. NAM= non-associatively tolerant animals administered morphine on experimental day 6.



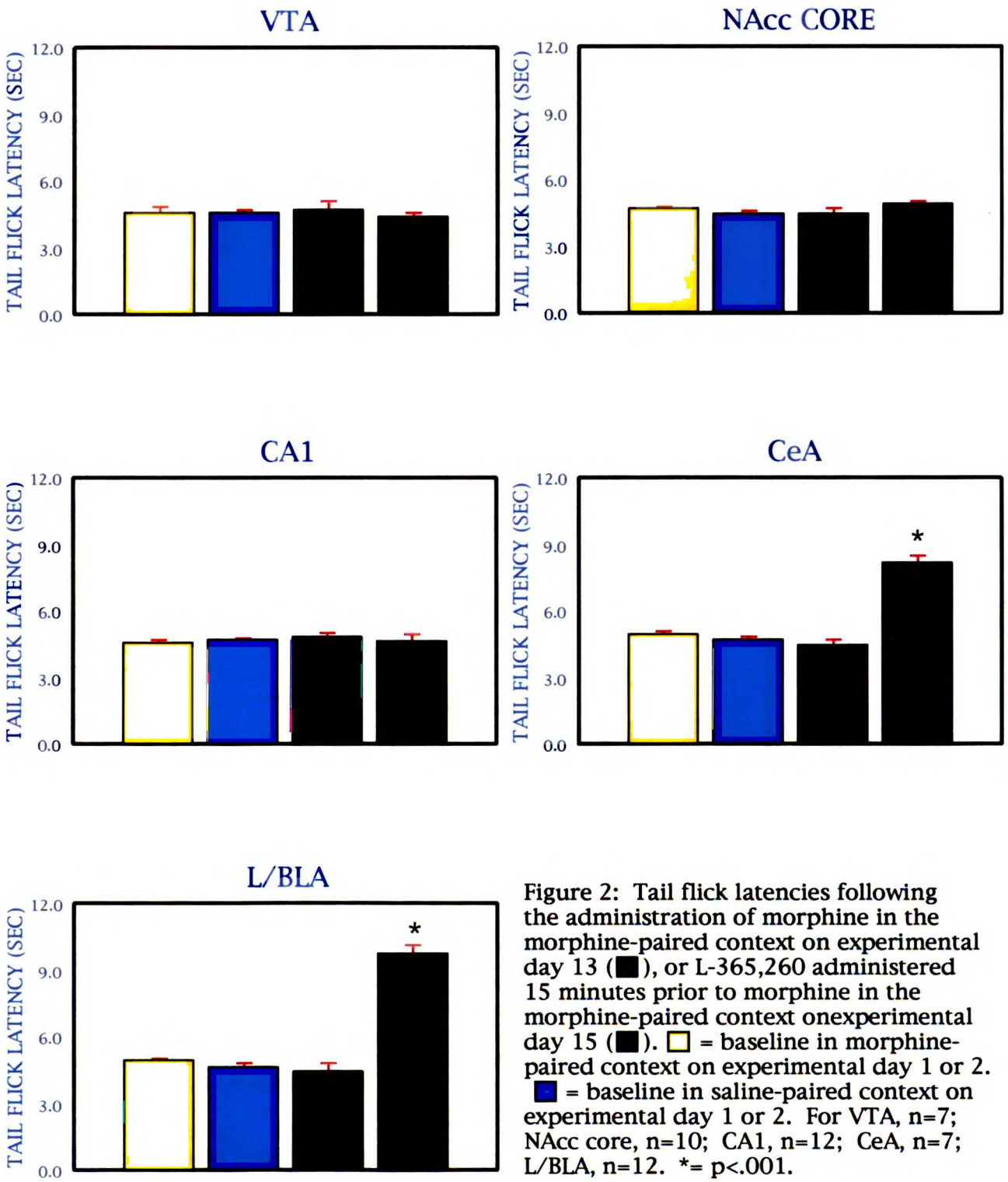
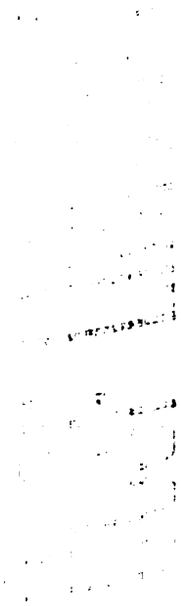


Figure 2: Tail flick latencies following the administration of morphine in the morphine-paired context on experimental day 13 (■), or L-365,260 administered 15 minutes prior to morphine in the morphine-paired context onexperimental day 15 (■). □ = baseline in morphine-paired context on experimental day 1 or 2. ■ = baseline in saline-paired context on experimental day 1 or 2. For VTA, n=7; NAcc core, n=10; CA1, n=12; CeA, n=7; L/BLA, n=12. *= p<.001.



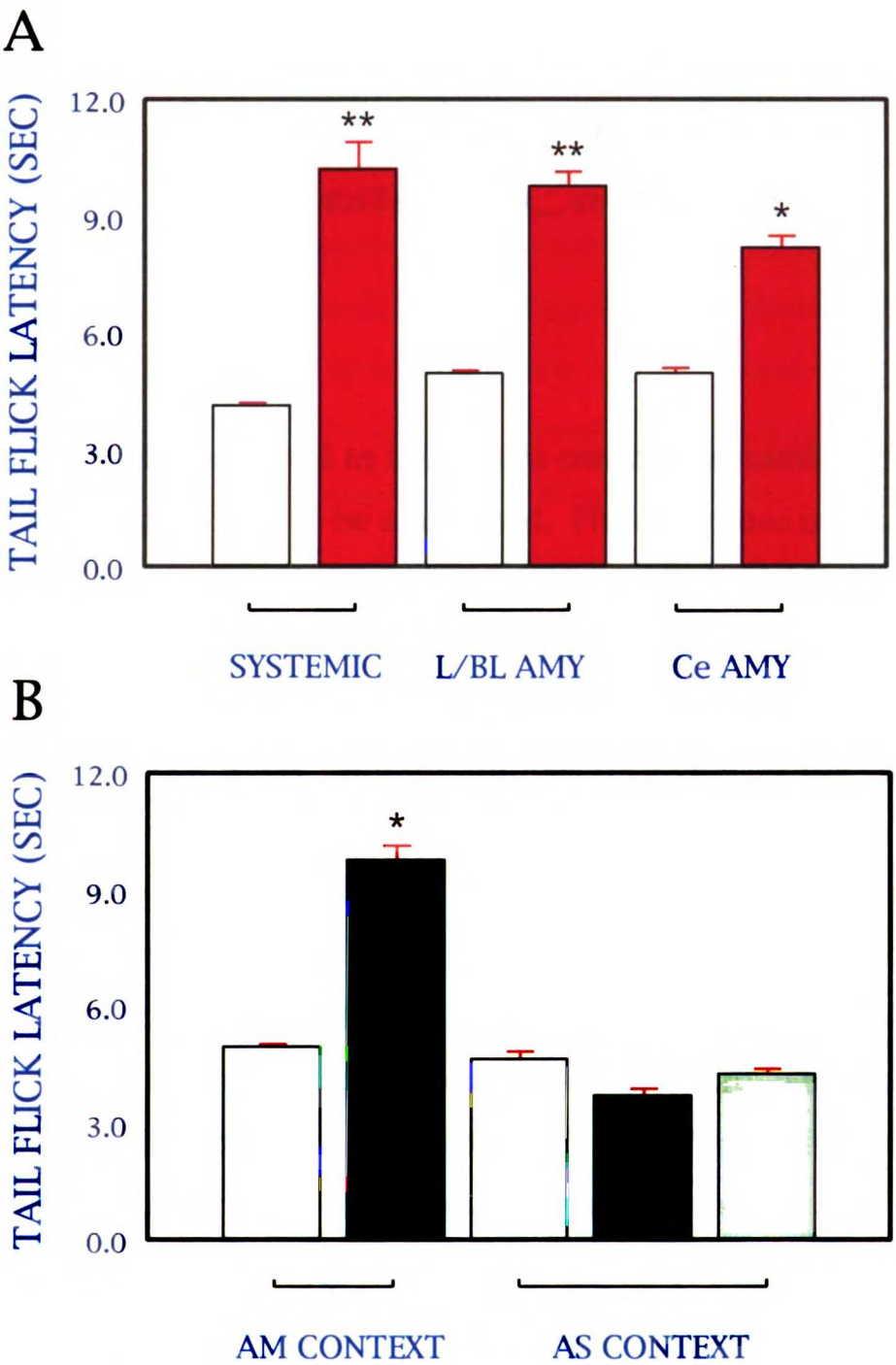


Figure 3A: Comparison of tail flick latencies following systemic administration of L-365,260 or microinjections of L-365,260 into the L/BLA or CeA. ☐ = baseline in the morphine-paired context on experimental day 1 or 2. ■ = L-365,260 administered 15 minutes prior to morphine administration in the morphine-paired context on experimental day 15. Systemic, n=11; L/BLA, n=12; CeA, n=7.

Figure 3B: Comparison of tail flick latencies following the administration of L-365,260 or vehicle in either the morphine-paired or saline paired-context. ☐ = baselines in the saline-paired (AS) and morphine-paired (AM) contexts. ■ = L-365,260 administered either 15 minutes prior to morphine in the morphine-paired (AM) context on experimental day 15 or 15 minutes prior to saline in the saline-paired context (AS) on experimental day 17. ☐ = vehicle (90% ETOH) administered 15 minutes prior to saline in the saline-paired context on experimental day 17.

Chapter 6:
Caveats and Conclusions

Caveats

The research presented in this thesis contains a number of short-comings which need to be addressed. First, because non-associative tolerance is produced via the s.c. administration of 75 mg morphine pellets, while associative tolerance is produced via the repeated i.v. administration of 2.5 mg/kg morphine, the sum dose of morphine that non-associatively tolerant animals receive over the course of an experiment is substantially higher than the sum dose received by associatively tolerant animals. Therefore, it is possible that the results obtained in the c-fos portion of this study are due to differences in the total dose of morphine administered and not to differences between associative and non-associative tolerance, per se. It would be possible to address this issue by administering morphine to associatively tolerant animals for a period of several months, rather than a period of two weeks, so that both associatively and non-associatively tolerant animals receive the same sum dose of morphine. However, this would have been an impossible scenario within the confines of the present study due to temporal, financial, and spatial considerations.

Another short-coming of this thesis is that route of drug administration is different between associatively and non-associatively tolerant animals. Therefore, it is also possible that

differences found between associative and non-associative animals are the result of route of drug administration and not the result of inherent differences between associative and non-associative tolerance. This issue could be addressed by administering morphine i.v., rather than s.c., to non-associative animals. However, when administering morphine i.v. one must be extremely careful to ensure that salient environmental cues (such as the injection procedure itself) are not predictive of drug administration in a way that an animal may learn to recognize. Additionally, in order to administer a large enough dose of morphine over a brief enough period of time to produce non-associative tolerance, animals would need to be injected i.v. every 2-6 hours for 72 hours. This was impossible in the current paradigm due to the necessity for sleep on the part of the experimenter.

Lastly, within this set of experiments it is impossible to differentiate between the effects of L-365,260 microinjected into the lateral versus into the basolateral amygdala. Because of the close proximity of these two nuclei and the rate of diffusion of the CCK-B antagonist, it is not possible to selectively microinject this compound into either of these two sub-nuclei. Similarly, it is impossible to say with certainly that L-365,260 is not having an effect in the central nucleus of the amygdala. These issues could be addressed by selectively chemically or electrolytically lesioning each of these three sub-nuclei prior to the expression of associative tolerance and the microinjection of L-365,260. However, such lesions are extremely

difficult to perform and would necessitate the use of a large number of animals.

Conclusions

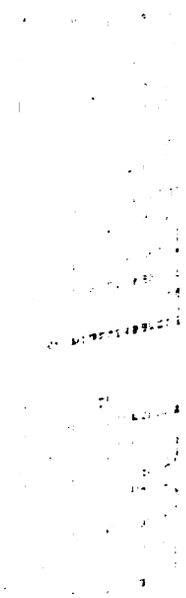
This thesis has attempted to demonstrate the importance of the anti-opioid peptide cholecystokinin (CCK) to morphine analgesia and tolerance. Additionally, it has sought to determine the contribution of CCK to conditioned learning. Several conclusion can be drawn from the research presented herein:

- When microinjected into the rostral ventromedial medulla (RVM), the anti-opioid peptide cholecystokinin can reverse the effects of systemically administered morphine in awake, unrestrained animals.

- The selective CCK-B antagonist, L-365,260 is able to reverse associative but not non-associative morphine tolerance. This effect is at least partially due to an action in the lateral and basolateral amygdala.

- Both the lateral and basolateral nuclei of the amygdala and area CA1 of the hippocampus are active following exposure to an environment that had been repeatedly paired with morphine administration.

When taken together, these results suggest that CCK is part of a learned compensatory response which serves to antagonize the



effects of repeated opioid administration through a mechanism which includes the lateral and basolateral amygdala.