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# The Role of Midbrain Substance P in Stress-Induced Analgesia using the Formalin Test for Tonic Pain

Nadège Altier

A Thesis

in

The Department

of

Psychology

Presented in Partial Fulfilment of the Requirements
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## ABSTRACT

The role of midbrain substance P in stress-induced analgesia using the formalin test for tonic pain

Nadège Altier

Footshock stressors induce both analgesia and activation of midbrain dopamine (DA) systems arising from the ventral tegmental area (VTA). Previous work has shown that activation of midbrain DA systems causes analgesia in a test for tonic pain. Because substance P (SP) in the VTA has been shown to be critical to this stress-induced activation of midbrain DA systems, a series of experiments were designed to test the analgesic effects of SP in the VTA. In a first set of experiments, microinfusions of the SP analogue, DiMe-C7, in the VTA induced analgesia in the formalin test for tonic pain and increased *in vivo* extracellular levels of DA and its major metabolite, dihydroxyphenylacetic acid (DOPAC), in the nucleus accumbens septi (NAS). When DA release was induced by amphetamine infusions in the NAS, but not the medial prefrontal cortex (mPFC), analgesia was also produced in the formalin test. In contrast, these manipulations of midbrain DA systems by SP and amphetamine caused, if anything, hyperalgesia in the tail-flick test for phasic pain. These findings suggest that activation of mesolimbic DA neurons innervating the NAS contributes to the suppression of tonic pain. A second set of experiments explored the possibility that SP release in the VTA mediates stress-induced analgesia in the formalin test. Intra-VTA blockade of NK-1 or NK-3 tachykinin receptors (with which SP and SP analogues interact) reversed, in part, footshock-induced analgesia. Furthermore, blockade of opioid receptors in the VTA prevented footshock-induced analgesia. These findings suggest that both opioids and tachykinins in the VTA are involved in the mediation of stress-induced analgesia in the formalin test. Because of problems with the

solubility of the tachykinin receptor antagonists used, these findings are preliminary, but suggest that the role of midbrain SP should be evaluated in both opioid- and non-opioid mediated stress-induced analgesia using nonpeptide compounds that can be dissolved for intracranial microinfusions.

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# INTRODUCTION

The response to pain, whether perceptual or behavioral, can be modulated by activity of the central nervous system. In recent years, the neuroanatomical and neurochemical systems that serve to modify the response to pain have received increasing attention. It has been found that the response to pain can be suppressed or inhibited by activity at several levels of the brain and spinal cord, and that pain, itself, can serve as a stimulus to activate these endogeneous mechanisms for the suppression of pain.

Until recently, most of the experimental evidence for endogeneous pain-inhibition mechanisms was provided by studies employing electrical stimulation of discrete brain regions. Reynolds (1969) was the first to report that, in rats, electrical stimulation of neurons in the periaqueductal grey of the midbrain produces sufficient analgesia to permit abdominal surgery without the use of anesthetic drugs. Brain stimulation produced analgesia was subsequently replicated in the rat (Mayer *et al.*, 1971; Mayer & Liebeskind, 1974) and extended to other species including the cat (Oliveras *et al.*, 1974), monkey (Goodman & Holcombe, 1976; Ruda *et al.*, 1976) and human (Adams, 1976). Detailed mapping studies performed in the cat and rat indicate that stimulation of sites located within the brainstem are most effective in producing analgesia (see Besson & Chaouch, 1987, for review). Central injections of opioid agonists and antagonists to discrete regions in the brainstem and spinal cord suggest that at least some of the effects induced by brain stimulation are mediated by actions at opioid receptors (Bodnar et al., 1988; Herz et al., 1970; Jacquet & Lajtha, 1973; Mayer & Murphin, 1976; Tsou & Jang, 1964; Yaksh & Rudy, 1977).

Studies such as these demonstrate the existence of endogeneous pain-inhibition mechanisms and have contributed to the understanding of the neural circuitry underlying brain stimulation produced analgesia and/or opioid analgesia (Advocat, 1988; Basbaum & Fields, 1984; Yeomans & Proudfit, 1992; Ma & Han, 1991). Unfortunately, these studies

involve unusual and invasive procedures and thus provide very little information about the ways in which pain-inhibition mechanisms are normally activated. The existence of endogeneous analgesic substrates suggests that, whereas pain serves an important biological purpose (Melzack & Wall, 1988), there are circumstances in which pain inhibition may be adaptive. In the last 15 years, much effort has been devoted to the search for environmental conditions that trigger the activation of these endogeneous mechanisms.

**Stress-Induced Analgesia**

Initial studies on environmentally-induced analgesia were carried out almost simultaneously in three separate laboratories (Akil *et al.*, 1976; Hayes *et al.*, 1976, 1978a,b; Rosecrans & Chance, 1976). It was found that analgesia could be produced by such diverse environmental stimuli as exposure to inescapable footshock given acutely (Akil *et al.*, 1976; Hayes *et al.*, 1976, 1978a,b) or chronically (Rosecrans and Chance,1976), to centrifugal rotation and to intraperitoneal saline injections (Hayes *et al.*, 1976, 1978a,b). These treatments appeared to affect pain responses specifically, since responses to tactile stimulation were unaffected (Hayes *et al.*, 1978a). Interestingly, it was also realized that, although all of the environmental manipulations found to induce analgesia were stressors (as defined by activation of the hypothalamus-pituitary-adrenal axis), not all stressors were effective; neither exposure to ether vapors nor horizontal oscillation induced analgesia in these studies (Hayes *et al.*, 1978b).

Since these initial observations, a wide variety of stressors have been reported to inhibit responses to painful stimulation. Some of these include cold (Bodnar *et al.*, 1978b) and warm water swimming (Vaccarino *et al.*, 1992a,b; Willow *et al.*, 1980), restraint (Amir & Amit, 1978), environmental novelty (Abbott *et al.*, 1986), aggressive confrontation and defeat with a conspecific (Miczek *et al.*, 1982, 1985), insulin injections (Bodnar *et al.*, 1979), exposure to a predator (Lester & Fanselow, 1985), hypoglycemia

(Bodnar *et al.*, 1978a), body pinch (Ornstein & Amir, 1981), food deprivation (Bodnar *et al.*, 1978), odors released by stressed conspecifics (Fanselow & Sigmundi, 1986), tail pinch (Levine *et al.*, 1982) and footshock (e.g. Chance *et al.*, 1977; Chance & Rosecrans, 1979; Cheser & Chan, 1977; Fanselow & Baackes, 1982; Fanselow & Bolles, 1979a,b; Hayes *et al.*, 1978b; MacLennan *et al.*, 1980; Ross & Randich, 1985; Watkins *et al.*, 1982). Exposure to stress has been observed to inhibit responses to a variety of noxious painful stimuli, such as application of heat to the tail (e.g. Cannon *et al.*, 1983; Drugan *et al.*, 1985; Lewis *et al.*, 1983) or paws (e.g. Amir & Amit, 1979; Blair *et al.*, 1982) and subcutaneous injections of formalin into a paw (Abbott *et al.*, 1986; Fanselow, 1984; Fanselow & Baackes, 1982; Fanselow *et al.*, 1988; Fanselow *et al.*, 1989a,b; Fanselow & Helmstetter, 1988; Fanselow & Sigmundi, 1986; Helmstetter, 1992; Helmstetter & Fanselow, 1987; Lester & Fanselow, 1985; Maier *et al.*, 1984; Vaccarino *et al.*, 1992 a,b).

*The neurochemical nature of stress-induced analgesia*

Early observations indicated that analgesia produced by exposure to stressful stimuli was similar to that induced by exogeneous opioids. These studies suggested that stress-induced analgesia might be mediated by the recently discovered endogeneous opioid peptides, endorphins, enkephalins and dynorphins (Akil et al., 1984; see Bodnar *et al.*, 1980, for review). Akil *et al.* (1976) and Chesher and Chan (1977), for instance, studied the analgesic effect of footshock stress and found that the opioid antagonist, naloxone, could block the response. In addition, other studies indicated that, like opioids, tolerance develops to the analgesic effects of repeated exposure to stress and cross-tolerance occurs between opioids and stress (Chesher & Chan, 1977; Lewis *et al.*, 1981; Terman *et al.*, 1986). Naloxone-sensitive stress-induced analgesia has also been reported in humans (Willer *et al.*, 1980). Other studies, however, found that, under some conditions, naloxone failed to reverse stress-induced analgesia (see Lewis, 1986, for

review), suggesting that at least two neurochemically discrete forms of stress-induced analgesia exist, one mediated by opioids and one that is not. Many studies followed in which the determinants of opioid and non-opioid stress-induced analgesia were sought using a single and reliable stressor, inescapable footshock stress.

Lewis *et al.* (1980) observed that exposure to footshock of a constant intensity could produce opioid or non-opioid forms of stress-induced analgesia depending on the temporal parameters of the stressor. More specifically, they found that 20-minute exposure to a 2.5 mA prolonged, intermittent footshock applied once every 5 seconds caused analgesia that was blocked by naloxone at a dose as little as 0.1 mg/kg, suggesting opioid involvement. In contrast, exposure to a 2.5 mA footshock applied continuously for 3 minutes caused equipotent analgesia which was unaffected by even high doses of naloxone. The naloxone-sensitive analgesia produced by prolonged, intermittent footshock also satisfies other criteria for opioid involvement. It shows complete tolerance after 14 days of exposure to this stressor (Lewis *et al.*, 1981; Mayer & Price, 1976) and shows cross-tolerance with morphine in that the analgesia produced by this stressor is markedly reduced in morphine-tolerant rats (Mayer & Price, 1976). The naloxone-insensitive analgesia produced by brief, continuous footshock, on the other hand, fails to manifest tolerance or cross-tolerance with morphine (Mayer & Price, 1976).

In summary, exposure to prolonged, intermittent footshock stress produces an opioid form of stress-induced analgesia whereas exposure to brief, continuous footshock stress produces a non-opioid form of stress-induced analgesia. Terman *et al.* (1984) subsequently extended these findings and observed that the neurochemical nature of stress-induced analgesia produced by continuous footshock depends on shock severity (intensity x duration). Based on their findings, these investigators suggested that the neurochemical basis of analgesia produced by exposure to continuous footshock stress follows a coulometric (intensity x duration) relation such that stress-induced analgesia is opioid-mediated if the product of these variables is below 7.5 (2.5-mA shocks for 3

minutes) but non-opioid if the coulometric product is at or above 7.5. Stress severity appears to play a similar role in determining the opioid vs non-opioid nature of stress-induced analgesia from cold water swim stress (Terman *et al.*, 1986) and from conditioned fear (Fanselow, 1984).

Several lines of evidence suggest that peptide-dopamine (DA) interactions in the midbrain might underlie stress-induced analgesia. Exposure to stressors such as footshock, conditioned fear and immobilization activates DAergic neurotransmission in neurons originating from the ventral tegmental area (VTA) of the midbrain and the release of opioid peptides in the VTA appears to be operative in this stress-induced biochemical response (Kalivas & Abhold, 1987). Recently, it has been reported that blockade of opioid receptors in the VTA prevents morphine analgesia in the formalin test for tonic pain, suggesting that systemic morphine produces analgesia in this test by causing the release of opioid peptides in the VTA (Morgan, 1990). The finding that morphine infused directly into the cell bodies of the VTA produces analgesia in this test (Franklin, 1990; Morgan, 1990) supports this view.

Release of the tachykinin neuropeptide substance P (SP) in the VTA has also been reported to play a critical role in the stress-induced activation of midbrain DA systems (Bannon et al., 1983). The evidence that stressors inhibit pain and cause SP-induced activation of midbrain DA systems and that analgesia can be induced by opioid-DA interactions suggests that SP release in the VTA might underlie stress-induced analgesia. The remaining sections will review in greater depth the evidence that SP interacts with midbrain DA neurons in response to stress and that it may underlie a form of stress-induced analgesia in the central nervous system (CNS). A clear understanding of the literature, however, requires an initial description of the anatomy of midbrain DA systems.

## Neuroanatomy of Midbrain Dopamine Systems

The greatest concentration of DA-containing neurons in the CNS lies in the midbrain. These neurons arise from the continuum of cell bodies located in the VTA, substantia nigra (SN) and retrorubral field and send axons ipsilaterally through the medial forebrain bundle to various forebrain sites (Dahlstrom & Fuxe, 1964). Subpopulations of midbrain DA ascending neurons are classified and referred to as mesocortical, mesolimbic and nigrostriatal, according to their site of origin and innervation. Mesocortical DA neurons arise from the VTA and project rostrally to innervate the prefrontal, entorhinal, anterior cingulate, and piriform cortices (Berger *et al.*, 1976; Fuxe *et al.*, 1974; Lindvall *et al.*, 1978; Thierry et al, 1973). Mesolimbic DA neurons arise from the VTA and project rostrally to various limbic structures including the NAS (also known as the ventral striatum), olfactory tubercle, medial portion of the lateral septum, amygdala, bed nucleus of the stria terminalis, anterior olfactory nuclei and, to a much lesser extent, the olfactory bulb and the nuclei of the diagonal band (Bjorklund & Lindvall, 1984; Swanson, 1982). The cells comprising the nigrostriatal pathway originate in the SN and project rostrally to innervate the striatum (which includes the caudate nucleus and the putamen) and the globus pallidus (Bjorklund & Lindvall, 1984). An important finding is that different portions of the VTA-SN cell groups give rise to different ascending projections (Fallon, 1988). For instance, whereas mesolimbic neurons innervating the olfactory tubercle and amygdala derive their cell bodies from the dorsal VTA, those innervating the NAS and lateral septum derive their cell bodies from the medial and ventral VTA, respectively. It is also important to keep in mind that cells located in both the VTA and SN contribute to mesocortical, mesolimbic and nigrostriatal ascending systems, although cells in either cell group are predominantly implicated in one system over the other (Fallon, 1988). For instance, the majority of mesocortical neurons innervating the frontal, anterior cingulate and suprarhinal cortices originate from the VTA but a minority of these also derive from

discrete portions of the SN. Similarly, the caudate nucleus is innervated primarily by nuclei located in the SN but it also receives a few inputs from the ventral portion of the VTA. More detailed reviews of the neuranatomy of midbrain DA systems are provided by Bjorklund and Lindvall (1984), Fallon (1988), Oades and Halliday (1987), and Fallon and Loughlin (1987).

Midbrain ascending DA systems play a cardinal role in the mediation of cognitive, emotional, motivational and motor processes. Activation of these systems can elicit a wide spectrum of goal-directed behaviours such as exploration, locomotion, sniffing and heightened attention to environmental stimuli (e.g. D'Angio et al; 1988; Joyce & Iversen, 1979; Kalivas *et al.*, 1983; Kelley *et al.*, 1985; Stewart, 1991; Stewart & Vezina, 1987, 1988, 1989; Stinus *et al.*, 1978; Vezina *et al.*, 1987, 1989; Vezina & Stewart, 1984, 1989, 1990). Another role recently ascribed to midbrain DA systems is, as previously mentioned, the mediation of analgesia (Morgan & Franklin, 1991). Finally, midbrain DA systems are also implicated in the pathophysiology of psychiatric (e.g. psychosis; Crow, 1980; Fuxe *et al.*, 1974; Kalivas & Stewart, 1991) and neurological (e.g Parkinson's and Huntington's disease; Crow, 1980) disorders.

**Interaction Between Stress and Midbrain DA Systems**

As previously mentioned, certain stressors such as mild intermittent footshock stress consistently activate DAergic transmission in midbrain ascending neurons, as reflected by increased levels of the DA metabolite dihydroxyphenylacetic acid (DOPAC; Glowinski, 1984; Roffler-Tarlov *et al.*, 1987; Roth *et al.*, 1976) in midbrain cell bodies and terminal fields. Thierry *et al.* (1976) offered the first evidence for this heterogeneous stress-induced activation of ascending DAergic neurons. They reported that exposure to mild intermittent footshock stress (1.6 mA) resulted in a pronounced increase in DA utilization in the PFC, and a smaller but significant increase in NAS DA utilization. These findings were subsequently confirmed by other investigators, using various footshock

parameters (Deutch *et al.*, 1985 b; Deutch *et al.*, 1990; Fadda *et al.*, 1978; Herman *et al.*, 1982; Lavieille *et al.*, 1978; Tissari *et al.*, 1979). Stressors other than exposure to footshock stress have similarly been documented to enhance DOPAC levels in the PFC and NAS. These include conditioned fear (Deutch *et al.*, 1985 b; Deutch & Roth, 1990; Herman *et al.*, 1982; Roth *et al.*, 1988), restraint (Deutch & Roth, 1990; Imperato *et al.*, 1991; Kennedy *et al.*, 1980; Roth *et al.*, 1988), tail pinch (Abercrombie et al., 1989; Bertolucci-D'Angio et al., 1990 a,b), swim stress (Knorr *et al.*, 1984; Yang *et al.*, 1985), forced locomotion (Bertolluci-D'Angio et al., 1990 a,b) and environmental novelty (Tassin *et al.*, 1980).

Whereas the PFC consistently responds to even minor forms of stress, the stress-induced biochemical activation mesolimbic DA neurons innervating the NAS appears to be more variable (Deutch *et al.*, 1985 b; Deutch *et al.*, 1990; Lavieille *et al.*, 1978; Roth *et al.*, 1988). Stress severity seems to play an important role in determining the profile of activation in various midbrain terminal regions. Increases in either intensity or duration of footshock stress enhance DA metabolism preferentially in the PFC followed by the NAS (Deutch *et al.*, 1990; Deutch & Roth, 1990; Roth *et al.*, 1988). For example, it has been found that exposure to 0.2 mA footshock stress increases DOPAC concentrations in the PFC only, whereas exposure to slightly more intense footshock (0.26 mA) increases this measure in the NAS (Deutch & Roth, 1990). Still, more severe stressors have been found to enhance DOPAC levels in striatal neurons (Cabib *et al.*, 1988; Deutch & Roth, 1990; Dunn, 1988; Roth *et al.*, 1988; Speciale *et al.*, 1986).

*Stress and SP*

Several findings indicate that SP release in the VTA plays a critical role in the stress-induced activation of ascending midbrain neurons. Bannon *et al.* (1983) found that pretreatment with a monoclonal SP antibody infused into the VTA completely antagonized the stress-induced activation of mesocortical neurons. In addition, it has been observed

that SP levels in the VTA are decreased during and following exposure to footshock stress, suggesting enhanced release of the peptide by the stressor (Bannon *et al.*, 1986; Deutch *et al.*, 1985a; Lisoprawski *et al.*, 1981). Finally, there is considerable indirect evidence that SP release in the VTA mediates the activation of midbrain DA neurons by stress. This indirect evidence is based on the finding that intra-VTA infusions of SP mimic the biochemical response induced by stress. The following section reviews this evidence in more detail.

**Interactions Between SP and Midbrain DA Systems**

Several studies have indicated that SP microinfused directly into the cell bodies of the VTA activates DA metabolism in mesolimbic and mesocortical neurons. For instance, Cador *et al.* (1989) found that intra-VTA SP infusions dose-dependently increase the DOPAC/DA ratio in the PFC and, to a lesser extent, in the NAS. Likewise, Deutch *et al.* (1985a) observed increased DOPAC levels in the PFC in response to intra-VTA SP infusions. Peripherally administered SP has also been shown to enhance DA neurotransmission in the NAS and striatum, as assessed by *in vivo* microdialysis (Boix *et al.*, 1992). Similar effects on DA metabolism in midbrain terminal fields have been reported following intra-VTA infusions of the enzyme-resistant and, therefore, long-acting SP analogue, DiMe-C7 (Elliott *et al.*, 1986).

Evidence for a close functional relationship between SP and midbrain DA systems is also provided by the finding that microinfusions of SP into the VTA augment DA-mediated behaviours. A number of studies have shown that DA agonists increase locomotor activity and that a primary event underlying this effect is the release of DA from terminals of mesolimbic neurons innervating the NAS. Stimulation of locomotor activity is produced by systemic administration of the indirect DA agonist amphetamine, the DA re-uptake blocker cocaine (Kelly & Iversen, 1976), the DA precursor L-DOPA, the direct DA receptor agonist apomorphine (Kelly *et al.*, 1975), and numerous other

DAergic-enhancing drugs (Cole, 1978; Isaacson *et al.*, 1978). Amphetamine-induced locomotor activity is greatly reduced by DA-depleting 6-hydroxydopamine (6-OHDA) lesions of the NAS (Kelly & Iversen, 1976; Kelly *et al.*, 1975) and by microinjections of the DA antagonist haloperidol in the NAS (Pijnenburg *et al.*, 1975), but is unaffected by a lesion to the PFC (Simon *et al*, 1981). Finally, it has been shown that microinjections of amphetamine, DA, or DA agonists directly into the NAS stimulate locomotor activity (Kelly, 1977; Pijnenburg *et al.*, 1976; Staton & Solomon, 1984), providing further evidence that an important mechanism underlying this behavioral response is DA release into the NAS.

Stinus *et al.* (1978) were the first to report that SP infusions into the VTA stimulate locomotor activity in rodents. This effect was subsequently replicated by other investigators (Kelley *et al.*, 1985; Kelley *et al.*, 1979) and also observed following intra-VTA infusions of the SP analogue, DiMe-C7 (Eison *et al.*, 1982 a,b; Elliott & Iversen, 1986; Naranjo & Del Río, 1984). SP and DiMe-C7-induced behavioural activation appears to be DA-dependent in that systemic administration of the DA antagonist, haloperidol, blocks the response (Eison *et al.*, 1982a; Naranjo & Del Rio, 1984), and that of the DA agonist, amphetamine, potentiates it (Eison *et al.*, 1982a; Stinus *et al.*, 1978). Pretreatment with either DA antagonist microinfusions or DA-depleting 6-OHDA lesions into the NAS blocks the behavioral response to SP, implicating the mesolimbic DA systems (Kelley *et al.*, 1979).

In summary, SP interacts with midbrain DA systems in a facilitatory way and plays an important role in the stress-induced activation of these systems. This is supported by the recent finding that SP terminals make direct synaptic contacts with DAergic neurons in the VTA (Tamiya *et al.*, 1990). Midbrain DA systems also appear to play a role in the mediation of analgesia (see Franklin, 1989, for review). The involvement of these systems in analgesia, however, has been shown to depend upon the type of pain test employed. Before this literature is considered, the following section describes the pain tests used in

such studies and then reviews the evidence that different neural systems mediate analgesia in different types of pain tests.

## Different Types of Pain Tests

Animal pain tests have been developed mainly to provide a screening device with which to test the analgesic effects of drugs. Over 50 different types of pain tests have been described in the literature and numerous variations of these have been used (Franklin & Abbott, 1989). The most commonly employed pain tests measure changes in the threshold at which a stimulus is first perceived as being noxious. The pain assayed by such tests is transient, short-lasting, rapidly rising and well-localized and thus has been termed 'phasic'. The escape response to phasic pain serves to prevent or minimize tissue damage and enduring pain. One of the most popular phasic pain tests, the tail-flick test (D'Amour and Smith,1941), measures the withdrawal response to thermal stimulation of the tail. The tail-flick response is organized at the level of the spinal cord, since it is elicited in spinally-transected rats (Carroll & Lim, 1960; Irwin *et al.*, 1951). Although studies employing phasic pain stimuli have provided considerable information concerning the neural mechanisms of pain and analgesia, the nature of the pain assayed in these tests does not ressemble that encountered in the clinical situation. Thus the findings derived from phasic pain tests provide limited information about the effectiveness of pain management techniques (e.g. analgesic drugs) on pain of pathological origin.

The formalin test was devised by Dubuisson and Dennis (1977) to provide a model of injury-produced continuous (i.e. tonic) pain similar to that encountered in the clinical situation. Unlike phasic pain tests, the formalin test assesses the behavioural recuperative responses to inescapable pain generated by a subcutaneous injection of dilute formalin into a rat's fore- or hindpaw. Pain responses last approximately 1½ hour and are characterized by favouring, raising and licking of the injured paw. Human volunteers who have sustained a formalin injection describe the pain as being poorly localized,

moderate in intensity, burning and throbbing with a time-course corresponding to the behavioural responses in animals (Alreja *et al.*, 1984; Dubuisson & Dennis, 1977; Franklin & Abbott, 1989). These qualities, except for burning sensations, are similar to those produced by postsurgical pain in humans (Franklin & Abbott, 1989). Together, these findings lend credibility to the idea that this test adequately serves its purpose as an animal model of clinical pain.

Formalin produces a distinct biphasic behavioral (Tjølsen *et al.*, 1992) and electrophysiological (Dickenson & Sullivan, 1987) response and there appear to be fundamental differences between the two phases. An early transient pain phase begins immediately following the formalin injection and is characterized by vigorous chewing, shaking, and licking of the injured paw. These pain responses subside 5 minutes later and give way to a period lasting 10-15 minutes in which an animal is relatively insensitive to the injury. A late pain phase begins approximately 20 minutes following the formalin injection, remains relatively steady for about 20 minutes and then gradually dissipates. Centrally-acting narcotic drugs such as morphine, codeine, meperidine, buprenorphine and pentazocine inhibit pain responses during both the early and late phase (Dubuisson & Dennis, 1977; Hunskaar *et al.*, 1986; Hunskaar & Hole, 1987; Shibata *et al.*, 1989; Vaccarino *et al.*, 1989). Several lines of evidence suggest that the early and late pain phases of the formalin test are mediated by independent physiological processes (see Tjølsen *et al.*, 1992, for review). The early pain phase appears to be due to direct stimulation of nociceptors and C-fibers since capsaicin pretreatment, which selectively destroys SP-containing unmyelinated sensory neurons, produces analgesia during the early but not the late phase (Shibata *et al.*, 1989). The late pain phase, in contrast, appears to result from ensuing inflammatory processes. Peripherally-acting non-steroidal (e.g. aspirin, indomethacin) and steroidal (e.g hydrocortisone, dexamethasone) anti-inflammatory drugs attenuate the late pain phase while leaving the early pain phase

unaffected (Hunskaar *et al.*, 1986; Hunskaar & Hole, 1987; Rosland *et al.*, 1990; Shibata *et al.*, 1989).

The neural structures involved in the mediation of formalin-induced pain responses appear to lie at ipsilateral forebrain sites. Unilateral knife cuts through the medial forebrain bundle, medial internal capsule and/or thalamus attenuate pain responses in the ipsilateral but not contralateral forepaw when both forepaws are injected with formalin (Amodei & Paxinos, 1980). More recently, it has been reported that the neural mechanisms underlying pain responses in the formalin test may lie at more caudal sites in the brainstem (Matthies & Franklin, 1990).

In summary, the formalin pain test differs from the more commonly used phasic pain tests, such as the tail-flick test, in that (1) pain in the formalin test is inescapable and long-lasting, whereas pain in phasic tests is escapable and short-lasting, (2) pain responses in the formalin test appear to be organized at forebrain sites, whereas those in phasic pain tests appear to lie at more caudal sites of the CNS and (3) pain responses in the formalin test are generated by tissue injury whereas those in phasic pain tests are produced by nondamaging stimuli.

The neural mechanisms underlying analgesia in the formalin test are not well understood, but are known to differ fundamentally from those involved in phasic pain tests. The following section reviews this literature.

**Different Neural Substrates Mediate Analgesia in Different Pain Tests**

Systemic morphine is believed to produce analgesia by acting synergistically at supraspinal brainstem and spinal sites. This view is supported by the finding that subanalgesic doses of morphine produce analgesia if administered concurrently in spinal and brainstem sites (Siuciak & Advocat, 1987; Yeung & Rudy, 1980). At supraspinal sites, morphine is thought to produce analgesia by activating spinally-projecting brainstem inhibitory pathways. According to one model (Basbaum & Fields, 1984), morphine

stimulates opioid receptors in the PAG of the brainstem. Neurons in the PAG project to serotonin-containing cells in the nucleus raphe magnus (NRM) and, in turn, to the dorsal horn of the spinal cord via the dorsolateral funiculus (DLF), where they inhibit neurons whose axons transmit pain messages to the CNS.

There is considerable evidence in support of this model (e.g. Basbaum & Fields, 1984; Bennett & Mayer, 1976; Bodnar *et al.*, 1988; Dickenson & Sullivan, 1986; Gebhart & Jones, 1988; Herz *et al.*, 1970; Jacquet & Lajtha, 1973, 1974; Mayer *et al.*, 1971; Mayer & Murphin, 1976; Mayer & Price, 1976; Tsou & Jang, 1964; Yaksh & Rudy, 1978). For instance, it has been reported that the analgesic effect of systemic morphine is reduced in spinally-transected animals relative to intact animals (Advocat, 1988). Presumably, this occurs because the spinally-projecting brainstem inhibitory systems are removed and the effect of systemic morphine is only expressed in the spinal cord. Recent data suggest that the role of brainstem descending mechanisms in opioid analgesia needs to be reinterpreted; it appears that morphine acts at brainstem sites to decrease, rather than increase, the activity of descending inhibitory pathways (Advocat, 1988; Advocat & Burton, 1987; Advocat & Gulati, 1991).

Despite the fact that the research on which the model proposed by Basbaum and Fields (1984) is based primarily relies on the use of phasic pain tests, inhibition of tonic pain is widely believed to occur through this brainstem descending inhibitory neurocircuitry. Several lines of evidence, however, suggest that different neural mechanisms are involved in producing analgesia when the nature of the pain is tonic.

Studies which examine the effects of lesions of the brainstem descending pain inhibitory systems indicate that they play a different role in the mediation of analgesia in phasic and tonic pain tests. For instance, lesions of the NRM, caudal PAG or DLF attenuate morphine analgesia in the tail-flick test but are without effect on morphine analgesia in the formalin test (Abbott & Melzack, 1982a; Abbott *et al.*, 1982b; Ryan *et al.*, 1985). Furthermore, while destruction of the median raphe nucleus has no effect on

morphine analgesia in the tail-flick test, it potentiates morphine analgesia in the formalin test. Finally, NRM lesions disrupt stimulation-produced analgesia (in the midbrain) in the tail-flick but not in the formalin test (Abbott & Melzack, 1983).

Pharmacological studies also reveal that the neurochemical systems subserving analgesia in phasic pain tests differ fundamentally from those involved in tonic pain tests. Drugs that deplete serotonin attenuate morphine analgesia in the tail-flick test but potentiate this effect in the formalin test (Dennis & Melzack, 1979). In contrast, drugs that enhance serotonergic activity potentiate morphine analgesia in the tail-flick test but diminish morphine analgesia in the formalin test (Abbott & Young, 1988). Franklin *et al.* (1990) obtained similar results in the clinical situation.

Studies that manipulate adrenergic systems reveal a similar dissociation of the neural mechanisms underlying analgesia between phasic and tonic pain tests. Dennis and Melzack (1980) reported that the alpha-adrenergic agonist clonidine enhances morphine analgesia in the formalin test but has no effect in either the tail-flick or the hot-plate test. In another study, clonidine was found to produce analgesia in both the formalin and the tail-flick test, but higher doses were required to elevate tail-flick latencies (Tchakarov *et al.*, 1985).

Support for the view that different neural systems mediate analgesia in different types of pain tests is also provided by the finding that tests which assay phasic vs tonic pain are differentially sensitive to the analgesic effects of opioid administration. Exogeneous opioids are more effective at alleviating tonic than phasic pain. In human volunteers, morphine is effective against continuous, intense, burning pain produced by exercising an ischemic limb (Smith *et al.*, 1966) but has little effect on phasic pain produced by non damaging stimuli such as pricking, pinching, or radiant heat (Beecher, 1968). These findings parallel those of clinical reports in which opioids relieve postoperative pain but do little, except at high doses, to suppress the twinges caused by the surgical wound (Dennis & Melzack, 1979; Jaffe & Martin, 1980).

As in the clinical situation, animal studies indicate that exogeneous opioids are more effective at alleviating tonic rather than phasic pain. Cohen *et al.* (1984) found that a relatively low dose range (2.5 - 10.0 μg) of morphine infused into the lateral ventricle produced analgesia in the formalin test whereas much higher doses (50.0 - 200.0 μg) were required to raise pain thresholds in the foot-flick test. In another study, it was found that morphine microinjected into the habenula produces analgesia in the formalin, but not in the foot-flick test (Cohen & Melzack, 1985).

The evidence that repeated administration of morphine differentially affects the analgesic response depending on the nature of the pain involved constitutes another line of evidence that different analgesia substrates are activated in different types of pain tests. When rats are exposed repeatedly to a given dose of morphine and tested for phasic pain, a typical finding is that morphine analgesia manifests tolerance (Adams *et al.*, 1969; Advocat, 1989; Bardo & Hughes, 1979; Kayan *et al.*, 1973; Mucha et al,1979; Siegel, 1975, 1976; Siuciak & Advocat, 1989; Stewart & Badiani, 1993). That is, the analgesic effect of a given dose of morphine declines progressively in response to repeated administration. Although phasic pain tests were used in these studies, health care professionals assume that the results derived from such studies apply to clinical pain and, therefore, believe that tolerance to morphine analgesia is likely to be a practical problem in the management of clinical pain (Melzack, 1990). As a result, the use of morphine for the management of clinical pain is not only limited but also ill-tailored to suit the needs for both pain relief and avoidance of aversive side-effects such as mental cloudness and nausea (Melzack, 1990). There is considerable evidence, however, to suggest that when opioids are taken on a long-term basis solely for the relief of clinical pain, tolerance to morphine analgesia is either nonexistent or minimal (Melzack, 1990; Isbell *et al.*, 1947; Mount *et al.*, 1976; Twycross, 1974, 1978) and the incidence of drug addiction is rare (Melzack, 1990). Abbott *et al.* (1981, 1982) took a closer look at the effect of repeated morphine administration on analgesia in the formalin test. In accordance with findings

obtained from clinical studies, they found that little tolerance develops to morphine analgesia in the formalin test whereas morphine analgesia in the tail-flick test shows rapid tolerance.

In summary, it appears that the neural substrates underlying inhibition of tonic pain differ from those underlying inhibition of phasic pain. This is supported by the findings that analgesia in these two types of pain is dissociable with respect to manipulations of serotonergic, noradrenergic, and opioidergic systems and with respect to the phenomenon of tolerance to morphine's analgesic effects. DA has also been found to play a different role in the mediation of analgesia in phasic and tonic pain. The following section reviews this evidence and introduces recent evidence which suggests that the neural substrates underlying inhibition of tonic pain may be located in midbrain DA systems.

**Role of Midbrain DA Systems in Analgesia**

Manipulations of DAergic activity produce different effects on pain responsiveness depending on the type of pain test employed. In the formalin test, DA agonists such as cocaine, amphetamine, apomorphine and quinpirole produce analgesia (Dennis & Melzack. 1983; Lin *et al.*, 1989; Morgan & Franklin, 1990; Skaburskis, 1980). The analgesic effect of cocaine is blocked by pretreatment with either the selective $D_1$ DA receptor antagonist SCH 23390 or the selective $D_2$ DA antagonist eticlopride (Lin *et al.*, 1989). Similarly, amphetamine-induced analgesia is blocked by pretreatment with either SCH 23390, the selective $D_2$ receptor antagonist pimozide, or the mixed $D_1$ and $D_2$ receptor antagonist cis-flupenthixol (Morgan & Franklin, 1991; Skaburskis, 1980). In contrast, when phasic pain tests are employed, DA agonists such as amphetamine, cocaine, bromocriptine and apomorphine have either no effect on withdrawal latencies or produce hyperalgesia (Ben-Sreti *et al.*, 1983; Carroll & Lim, 1960; Dennis & Melzack, 1983; Dunai-Kovács & Székely, 1977; Gonzales *et al.*, 1980; Hernandez *et al.*,1986; Misra *et*

*al.*, 1987; Nott, 1968; Pertovaara *et al.*, 1988; Robertson *et al.*, 1981; Tocco & Maickel, 1984; Tocco *et al.*, 1985; Tulunay *et al.*, 1976; Witkin *et al.*, 1961).

Inferences from the reward literature led Morgan and Franklin (Franklin, 1989; Morgan, 1990; Morgan & Franklin, 1990) to speculate that midbrain DA systems might be implicated in mediating the analgesic effects of drugs such as morphine and amphetamine in the formalin test. According to these investigators, reward and analgesia (more specifically, inhibition of tonic pain) are associated behavioural phenomena, in that both are the result of mood-altering psychological processes. For instance, in the case of analgesia, clinical studies indicate that morphine causes 'dissociative' analgesia by alleviating the suffering that accompanies chronic pain without reducing the sensory dimension of pain. Based on this proposed association between reward and analgesia, it was reasoned that knowledge of the neural systems underlying reward (induced by drugs or brain stimulation) might provide clues to the neural systems underlying inhibition of tonic pain. Because midbrain DA systems are involved in mediating reward (e.g. Bozarth, 1987; Bozarth & Wise, 1986; Broekkamp *et al.*, 1976, 1979; Fibiger & Philipps, 1989; Jenck *et al.*, 1987; Phillips & LePiane, 1980; Smith *et al.*, 1985; Spyraki *et al.*, 1982; Stewart, 1984; Stewart *et al.*, 1984; Wise, 1988; Wise & Bozarth, 1987; Yokel & Wise, 1975, 1976), these systems were likely candidates.

Morgan and Franklin (1990) initiated an experiment to investigate the role of midbrain DA systems on drug-induced analgesia in the formalin and, for comparison, the tail-flick test. In this study, they found that DA-depleting 6-OHDA lesions of the VTA abolish the analgesic effects of systemic morphine and amphetamine in the formalin test, but not in the tail-flick test, suggesting that midbrain DA systems are involved in the mediation of analgesia in the former but not the latter pain test. In another study (Franklin, 1989; Morgan, 1990), they reported that activation of midbrain DA systems by morphine microinfusions into the VTA induces analgesia in the formalin test, providing more direct evidence that these systems are implicated in analgesia in this test. Other data

obtained from the same laboratory indicate that 6-OHDA lesions of the NAS abolish amphetamine analgesia in the formalin test (Clarke & Franklin, 1992)..

**Rationale of the Present Experiments**

The findings that stressors inhibit pain and cause SP-induced activation of midbrain DA systems and that analgesia can be induced by the activation of these systems suggest that SP release in the VTA might underlie stress-induced analgesia. In particular, the evidence that midbrain DA systems are involved in supressing tonic but not phasic pain suggests that, to the extent that midbrain SP might underlie stress-induced analgesia, it is more likely to be implicated in this response in the formalin test for tonic pain than in a phasic pain test, such as the tail-flick test.

The purpose of Experiments 1 to 5 was to gain additional evidence in support of the idea that midbrain SP might underlie stress-induced analgesia in the formalin test. Thus, Experiment 1 was designed to test the analgesic effect of intra-VTA microinfusions of SP in the formalin test. Experiments 2 and 3 were designed to explore the involvement of midbrain DA terminal fields (i.e. the mPFC and NAS) in SP-induced analgesia in the formalin test. The effects observed in the formalin test (in Experiments 1 and 3) were compared, in Experiments 4 and 5, to those in the tail-flick test . Finally, the last series of experiments were designed to explore the role of midbrain SP on stress-induced analgesia in the formalin test. This was accomplished by examining the effect of two tachykinin receptor (with which SP and SP analogues interact) antagonists applied to the VTA on footshock stress-induced analgesia in the formalin test.

## THE EXPERIMENTS

### General Methods and Procedures

Because most of the experiments described in this thesis were conducted following the same basic procedures, these are described in the following section.

*Subjects, housing and habituation*

Naive male Wistar rats weighing 350-375 g (Charles River Canada, St-Constant, Quebec) served as subjects. Upon arrival, they were housed two by two in standard clear plastic shoebox cages with wire tops. Rats had continuous access to food and water and were maintained on a 12 h light-dark cycle (lights on at 9:00 PM and off at 9:00 AM). All testing took place in an illuminated test room during the dark portion of the light-dark cycle. On the four days prior to testing, each rat was handled for one minute. On the two days prior to either formalin or tail-flick testing, each animal was handled and habituated to the various testing procedures exactly as it was to be treated on the test day, except that neither intracranial nor formalin injections were administered.

*Surgery*

Seven to ten days after arrival, rats were anesthetized with an injection of sodium pentobarbital (Somnotol, 65 mg/kg, i.p.; MTC Pharmaceuticals Ltd., Cambridge, Ontario). When required, anesthesia was maintained with Methoxyflurane (Metofane; Pitman-Moore, Missisauga, Ontario). Following immobilization of the animals in a stereotaxic frame, an incision was made along the midline and the skull was exposed. Guide cannulae (Plastics One, Inc.) were implanted, bilaterally, 1.0 mm above the target structure and anchored to the skull with 4 to 5 stainless steel screws and dental acrylic cement. Prior to surgery, animals were injected with 0.6 mg/kg given s.c. of atropine sulfate (Glaxo Laboratories, Montreal, Quebec) and 0.1 ml given i.m. of Penicillin G (Ayercillin; Ayerst,

Montreal, Canada). Following surgery, 28 gauge stainless steel obturators (Plastics One, Inc.) were inserted into the guide cannulae. Unless otherwise specified, these extended 1.0 mm beyond the tip of the guide cannulae. Upon recovery under a heat lamp, rats were housed individually in standard plastic shoebox cages with wire tops and were allowed a 7-day recovery period before habituation to testing procedures was begun.

*Intracranial microinjections*

After removing the obturators, 28 gauge stainless steel internal injector cannulae extending 1.0 mm beyond the tip of the guide cannulae were inserted and held in place in the guide cannulae by a brass screw cuff. The injector cannulae were connected via polyethylene tubing to 1-µl Hamilton syringes. Compounds were administered in unrestrained rats in a volume of 0.5 µl/side over 60 seconds. The injectors remained in place for an additional 120 seconds to allow diffusion of the solutions around the injection site. Obturators were immediately replaced following the injection. To prevent intracranial infections, all internal injector cannulae and obturators were wiped with 70% alcohol and dried immediately prior to being inserted into the guide cannulae. On the two days prior to testing, each rat was habituated to the intracranial microinjection procedure. The above procedure was followed except that no injections were administered and the internal injector cannulae did not extend beyond the tip of the guide cannulae. In all experiments, the observer was blind to which animal had been injected with a drug and which with the vehicle.

*Histology*

Following completion of the experiments, rats were deeply anesthetized with chloral hydrate (1 ml, i.p.) and perfused transcardially with the injectors in place with 0.9 % saline followed by 10% formalin. Brains were then removed and stored in 10% formalin for at least one week. Histological verification of cannulae tip placements was subsequently determined on 30 micron thionine-stained coronal sections. Data from an

animal were included in the analyses only if the injector tips were located within 0.5 mm of the target structure.

*The Formalin Test*

On the two days prior to testing, each rat was habituated to the test cubicle by being placed in it and left undisturbed for at least 75 minutes. On the test day, pain responses were engendered by a subcutaneous injection of 0.05 ml of 2.5 % formalin into the plantar surface of one hind paw and were subsequently recorded once every 5 seconds using a time sampling procedure. Thus, 12 observations were made per minute. The intensity of pain was rated on a scale of 0 to 3 : a score of 0, if the rat walked or sat normally with weight placed equally on both hind paws; 1, if the rat favored the injured paw (e.g., if it limped); 2, if the rat held the injured paw off the floor, with at most the nails touching the floor; and 3, if the rat chewed or licked the injured paw. Mean formalin pain scores were determined for each 3-min period. If an animal failed to exhibit pain behavior at any time throughout a session, its data were discarded. Rats used in Experiments 1 and 3 were tested twice in the formalin test, at an interval of one week. The side of the paw injected was counterbalanced across rats and alternate hindpaws were injected with formalin on successive tests. In Experiments 2, 6, 7 and 8, rats were tested only once and the side of the paw injected with formalin was counterbalanced across rats. In all experiments, rats were tested two at a time except in Experiment 3, where rats were tested three at a time.

*The Tail-Flick Test*

On the 2 days prior to testing, rats were administered 7 habituation trials to the tail-flick test apparatus. On each of these trials, rats were hand-held for approximately 2 minutes on the platform, with their tails hanging over the side of the water tank. The bath circulator was in operation during habituation trials so as to allow the rats to adapt to its noise. On the test day, each rat was first given 2 habituation trials. Following these, rats received bilateral intracranial infusions of the appropriate compounds and tail-flick tests

were begun immediately once every 10 minutes for 1 hour. A test trial consisted of hand-holding a rat on the platform and immersing the distal 5-10 cm of the tail in the 55° C water. The latency for the animal to flick its tail out of the water was recorded. Rats were returned to their holding cages immediately after execution of a tail-flick. A cut-off time of 15 seconds was employed in order to avoid tissue damage. Rats were run in rotation, two at a time, and were tested twice with four days between tests.

## EXPERIMENT 1

### Effects of Intra-VTA Infusions of the SP Analogue, DiMe-C7, in the Formalin test.

The purpose of the Experiment 1 was to examine the analgesic effects of intra-VTA infusions of the SP analogue, DiMe-C7, in the formalin test. DiMe-C7 is a carboxy-terminal (SP $_{5-11}$) fragment of the SP molecule (Sandberg *et al.*, 1981). This compound is biologically active and resistant to enzymatic degradation in the CNS (Eison *et al.*, 1982b; Lee *et al.*, 1981; Sandberg *et al.*, 1981). When infused into the VTA, DiMe-C7 is equipotent with SP in causing locomotor hyperactivity, but the effects of DiMe-C7 are longer-lasting (Eison *et al.*, 1982a,b; Elliott & Iversen, 1986). Because of these qualities, DiMe-C7 has been proposed to be better suited for behavioral studies (Eison *et al.*, 1982a; Elliott & Iversen, 1986), hence its use in the present experiments.

As previously mentioned, formalin produces a distinct biphasic behavioral response characterized by an early and a late pain phase. It is generally thought that the nature of the pain in these two phases is qualitatively different in that the early phase is comparable to phasic pain whereas the late phase is comparable to tonic pain (Acton *et al.*, 1992). Another purpose of Experiment 1, therefore, was to compare the analgesic effect of intra-VTA DiMe-C7 infused immediately prior to either the early acute or the late tonic pain phase.

#### Method

*Apparatus*

The formalin test cubicles were made of clear Plexiglas and the dimensions were 30 x 30 x 30 cm. A mirror was mounted beneath the floor of the cubicle at a 45 ° angle to allow an unobstructed view of the rats' paws.

*Surgery*

21 mm long, 22 gauge guide cannulae were implanted, bilaterally, 1.0 mm above the VTA at the following coordinates : - 3.8 mm caudal from bregma, + 0.6 mm lateral

from midline, and - 7.5 mm ventral from the skull surface (Pellegrino *et al.*, 1979). To determine the site-specificity of the effects of DiMe-C7, a group of rats was implanted with bilateral cannulae aimed 2.0 mm dorsal to the VTA.

*Drugs*

(*p*-Glu$^{5}$-MePhe$^{8}$-MeGly$^{9}$) SP $_{5-11}$ (DiMe-C7; Sigma, St. Louis, MO) was infused bilaterally at a dose of 3.0 µg/0.5 µl/side. The compound was dissolved in acid saline (pH = 6.05) in order to inhibit absorption of the peptide to plastic. Stock concentrations (30.0 µg/5.0 µl) of DiMe-C7 and the vehicle, acid saline, were aliquoted into polypropylene vials and frozen at -70 ° C until used. Solutions were thawed within 30 minutes of use.

*Design and Procedure*

On the test day, animals received bilateral intra-VTA infusions of 3.0 µg/0.5 µl/side of DiMe-C7, or the vehicle, using a counterbalanced within-subjects design, either immediately prior to or 25 minutes following a subcutaneous injection of formalin into the plantar surface of one hind paw. Pain responses were continuously recorded for 75 minutes.

*Statistical Analyses*

Data were analyzed by two-way ANOVAs with repeated-measures on both Treatment (DiMe-C7 vs vehicle) and Time (blocks of 3 minutes) variables. The levels of the Time variable differed depending on whether DiMe-C7 was infused prior to or 25 minutes following the formalin injection. These analyses were followed, if appropriate, by simple main effects tests.

## Results

Figure 1 shows mean formalin pain scores of animals administered either DiMe-C7 or the vehicle immediately prior to a formalin injection over the course of the 75-minute observation session. Vehicle-treated animals typically exhibited biphasic pain responses. An early pain phase occurred during the first 5 minutes (data not shown) and

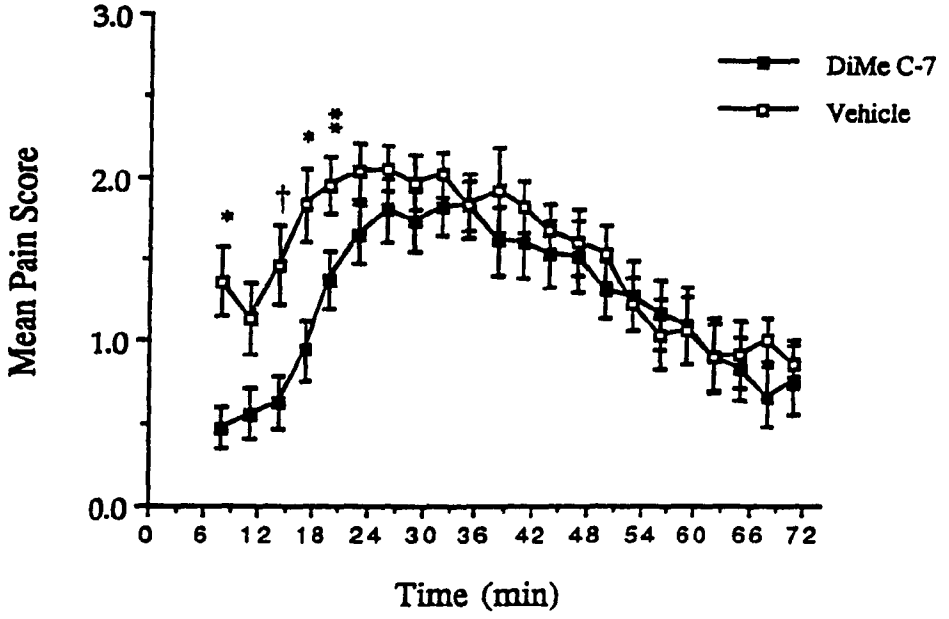


Figure 1. Effect of bilateral intra-VTA infusions of 3.0 μg/0.5 μl/side of DiMe-C7, or the vehicle, on formalin pain scores. Each time point represents an average formalin pain score (± S.E.M.) for a 3 minute period. Animals (n=11) were tested twice in a counterbalanced within-subjects design. Mean pain scores differed significantly between DiMe-C7 and vehicle at several of the early time points (* $p < 0.005$; † $p < 0.0125$; ** $p < 0.05$).

was followed by a period of less sensitivity to the injury. A later pain phase began approximately 20 minutes after the formalin injection, remained relatively steady for about 20 minutes and then gradually dissipated until the end of the session. Although DiMe-C7-treated rats also displayed biphasic pain responses, these were greatly attenuated during the first 25-30 minutes as compared to the vehicle condition. There was a significant Treatment x Time interaction, $F(27,270) = 2.52$, $p < 0.001$. Simple main effects tests indicated that at all times during the first 20 minutes, except at 11 minutes, the differences between DiMe-C7 and vehicle pain scores were significant. The location of the internal injector cannulae tips of rats administered DiMe-C7, or the vehicle, immediately prior to a formalin injection is illustrated in Figure 2. This figure shows that 11 rats had their internal injector cannulae tips within the VTA. The data for 2 rats were discarded because their internal injector cannulae tips were outside the limits of the VTA.

As shown in Figure 3 A, when DiMe-C7 was administered 25 minutes following a formalin injection, pain responses were attenuated for at least 30 minutes. There was a significant main effect of Treatment, $F(1,8) = 11.8$, $p < 0.01$, and a significant interaction between Treatment and Time, $F(21,168) = 5.4$, $p < 0.0001$. Simple main effects tests indicated that the differences between DiMe-C7 and vehicle were significant at all times following the intracranial infusions, except at 57 and 69 minutes. It can also be seen from Figure 3 A that prior to the intracranial infusions, all rats behaved similarly. Figure 3 B shows the data from rats injected with either DiMe-C7, or the vehicle, 1.0 mm dorsal to the VTA. There were no differences in pain responses between the two conditions either prior to or following the intracranial infusions, $F(1,3) = 0.14$, $p = 0.84$, nor was there a significant Treatment x Time interaction, $F(22,66) = 0.88$, $p = 0.96$. The location of the internal injector cannulae tips of rats administered DiMe-C7, or the vehicle, 25 minutes following a formalin injection is illustrated in Figure 4. As shown, 9 rats had their internal injector cannulae tips within the VTA and 4 rats in the control infusion group had

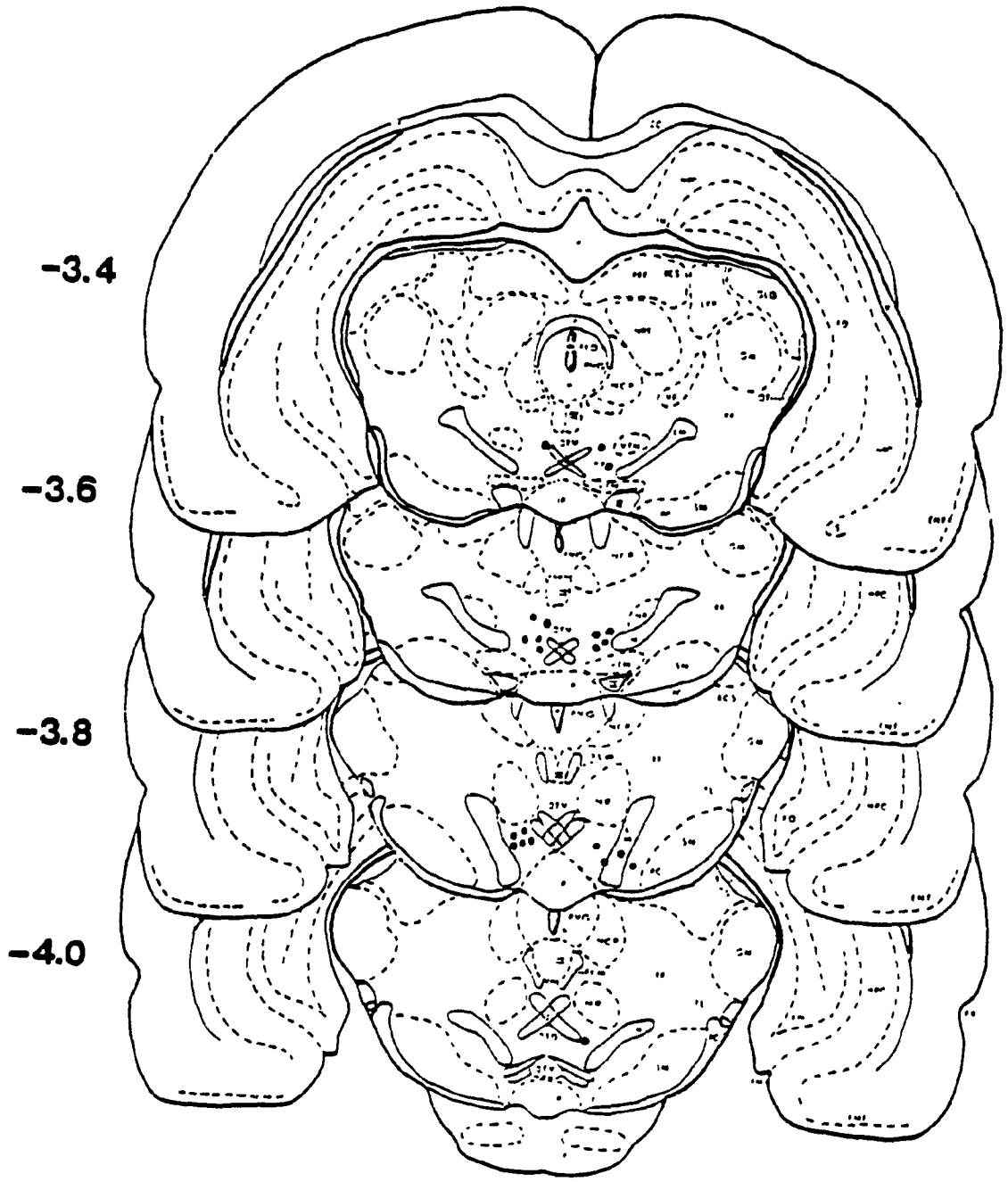


Figure 2. Location of the internal injector cannulae tips in the VTA of rats that received intra-VTA infusions of DiMe-C7, or the vehicle, immediately prior to the formalin injection. Drawings are from the atlas by Pellegrino *et al.* (1979).

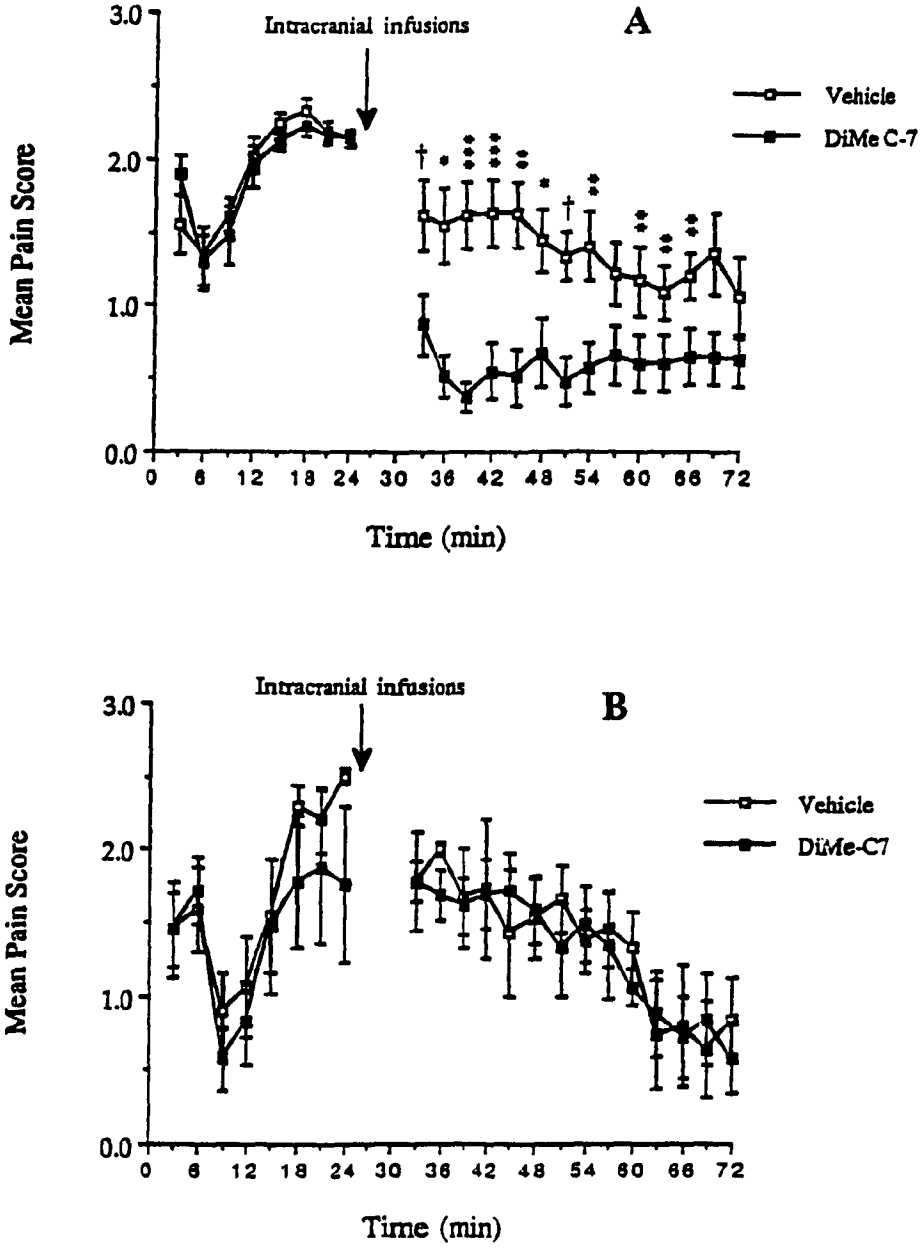


Figure 3. (A) Effect on mean pain scores (± S.E.M.) of bilateral intra-VTA DiMe-C7 (3.0 µg/0.5 µl/side), or the vehicle, infused 25 minutes following a formalin injection. Animals (n=9) were tested twice in a counterbalanced within-subjects design. Mean pain scores between DiMe-C7 and vehicle differed significantly at all times following the intracranial infusions, except at 57 and 69 minutes (** $p < 0.05$; † $p < 0.0125$; *** $p < 0.005$; * $p < 0.0025$). (B) Effect of DiMe-C7 (3.0 µg/0.5 µl/side), or the vehicle, infused bilaterally 1.0 mm dorsal to the VTA on mean formalin pain responses (± S.E.M.). Animals (n=4) were tested in a counterbalanced within-subjects design.

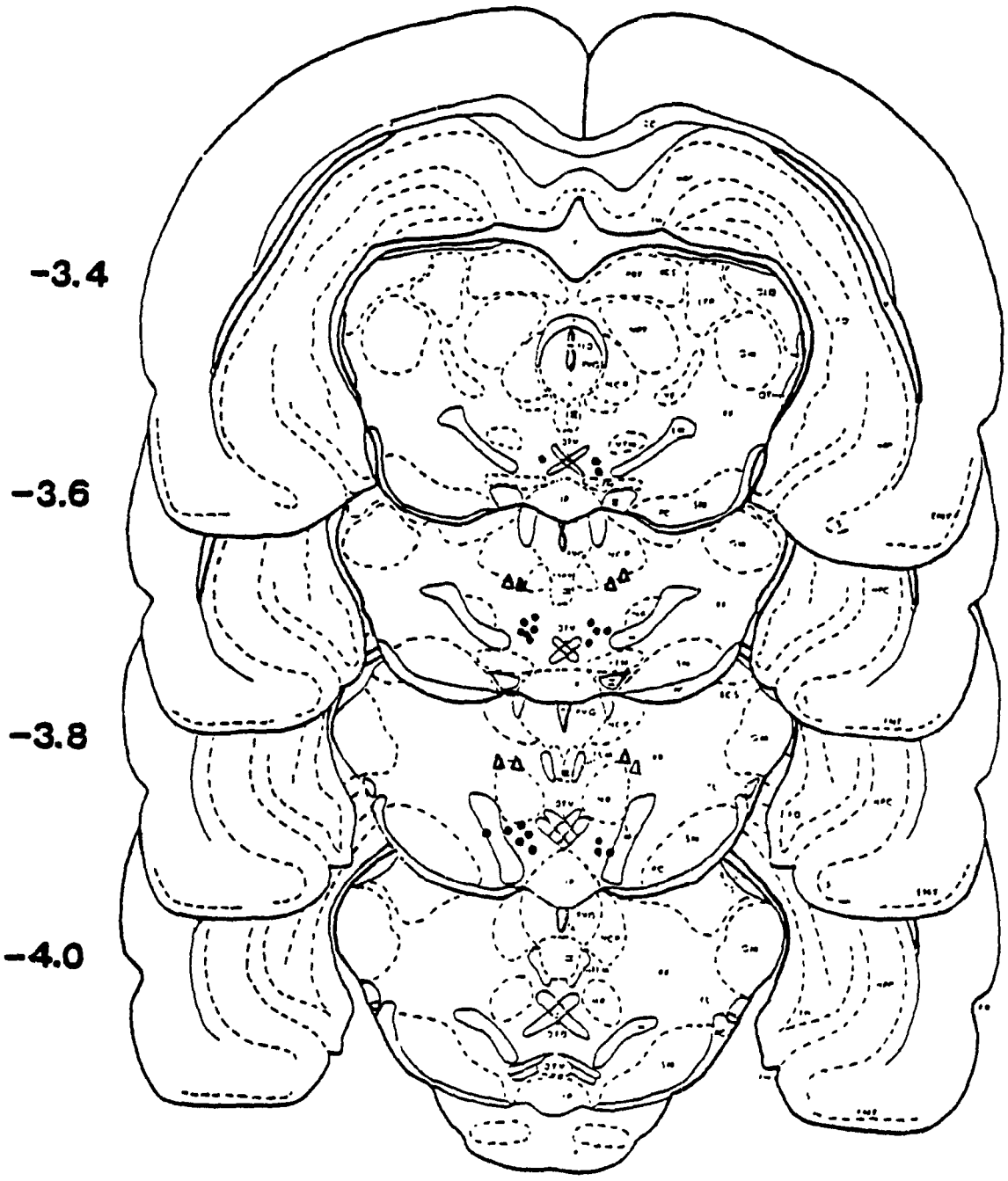


Figure 4. Location of the internal injector cannulae tips of rats that received infusions of DiMe-C7, or the vehicle, in the VTA (circles) or 1.0 mm dorsal to the VTA (triangles) 25 minutes following a formalin injection. Drawings are from the atlas by Pellegrino *et al.* (1979).

their injector tips 1.0 mm dorsal to the VTA. Four rats had their injector tips outside the limits of the VTA; their data were discarded.

## Discussion

The results obtained from the present experiment indicate that intra-VTA infusions of the SP analogue, DiMe-C7, cause analgesia in the formalin test for tonic pain and suggest that activation of midbrain DA systems may be involved in this response. The present results also indicate that DiMe-C7-induced analgesia is more potent and longer-lasting when the neuropeptide is infused just prior to the later and longer pain phase than it is when given just before the early acute pain phase, suggesting that the effect is more selective for tonic pain. DiMe-C7 appears to induce analgesia in the formalin test by acting upon receptors within the VTA since control infusions of the neuropeptide 1.0 mm dorsal to the VTA failed to produce this response. These results are consistent with those of previous studies showing that midbrain DA systems play a role in the mediation of analgesia in the formalin test (Clarke & Franklin, 1992; Franklin, 1989; Morgan & Franklin, 1990).

SP has been reported previously to increase nociceptive thresholds in phasic pain tests following intraventricular (Frederickson *et al.*, 1978; Kotani *et al.*, 1981; Malick & Goldstein, 1978; Mészaros *et al.*, 1980; Naranjo *et al.*, 1982a; Stewart *et al.*, 1976) and peripheral (Hall & Stewart, 1983; Mohrland & Gebhart, 1979; Oehme *et al.*, 1980; Starr *et al.*, 1978; Szreniawski *et al.*, 1979) administration. Recently, Yeomans & Proudfit (1992) reported that SP microinfused into the spinally-projecting noradrenergic A7 cell group increases nociceptive thresholds in the foot withdrawal test. A7 neurons receive afferent input from SP-containing neurons originating in the ventromedial medulla and appear to participate in the endogeneous descending pain-suppression system (Yeomans & Proudfit, 1991, 1992). The present findings indicate that SP can act independently in the VTA to suppress the transmission of tonic pain. There are earlier reports that SP infused

intraventricularly causes analgesia in the vocalization after-discharge test (Del Río *et al.*, 1983; Naranjo *et al.*, 1982), a test that has been proposed to be comparable to the formalin test in being dependent on forebrain structures and associated with significant negative affect (Morgan, 1990; Morgan & Franklin, 1990). Given the proposed parallels between these two pain tests, the present finding suggests that SP may similarly produce analgesia in the vocalization after-discharge test by acting within the VTA. SP has long been considered to be a transmitter in afferent fibers carrying information about painful stimuli. It is interesting to speculate that the same transmitter may be actively involved in pain- and stress-induced inhibition of pain. As part of the normal response to pain and stress, SP may be released in a number of different sites where it activates a number of independent pain-inhibitory circuits, in concert.

Recent data suggest that the development of the late pain phase depends upon the presence of the early pain phase. Vaccarino et al (1992a), for example, found that blocking the early phase by swim stress-induced analgesia prevents the manifestation of the late pain phase, whereas the same stressor given after the early phase was without effect on the late phase. Similar findings have been obtained with the use of local (Coderre *et al.*, 1990) or spinal (Coderre *et al.*, 1990; Dickenson & Sullivan, 1987) anesthesia and it has been hypothesized that neural changes generated during the early phase, in addition to local inflammation, are responsible for the development of the late pain phase. In contrast to the above findings, the present results indicate that (1) the manifestation of the late phase is not prevented by blockade of the early phase with intra-VTA DiMe-C7 and that (2) intra-VTA DiMe-C7 infused 25 minutes after the formalin injection blocks the development of the late phase despite the expression of the early phase. The present results, therefore, do not support the view that the development of the late phase depends upon the presence of the early phase. The discrepancy between the present and previous results may be attributable to the different times at which the manipulations were introduced. In the study mentioned above, for example, rats were

exposed to swim stress immediately following the early phase (5 minutes after the formalin injection) whereas, in the present study, rats were given intra-VTA DiMe-C7 a long while after the early phase (25 minutes after the the formalin injection).

## EXPERIMENT 2

## Effects of Intra-VTA Infusions of the SP Analogue, DiMe-C7, on Extracellular Dopamine in the Nucleus Accumbens.

Indirect evidence suggests that one way in which intra-VTA DiMe-C7 might produce analgesia in the formalin test is by activating DA neurotransmission in mesocorticolimbic neurons. As previously reviewed, intra-VTA SP and DiMe-C7 augment DA metabolism in the PFC and NAS (Cador *et al.*, 1989; Deutch *et al.*, 1985a; Elliott *et al.*, 1986) and it has recently been reported, using *in vivo* microdialysis, that peripherally administered SP or DiMe-C7 elevates extracellular DA in the NAS (Boix *et al.*, 1992 a, b). Other studies indicate that the locomotor stimulant effects of intra-VTA SP may be mediated by DA release from terminals of mesolimbic neurons innervating the NAS (Kelley *et al.*, 1979). The purpose of the present experiment was to examine more directly the effect of intra-VTA DiMe-C7 infused just prior to the late pain phase of the formalin test on extracellular NAS levels of DA and its metabolites, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) as well as on extracellular NAS levels of the serotonin metabolite, 5-hydroxyindoleacetic acid (5-HIAA). This was accomplished using microdialysis (Ungerstedt, 1984) in the NAS of the freely moving rat. In addition, locomotor activity was monitored throughout the test period.

### Method

*Apparatus*

Animals were tested in 42 x 39 x 33.5 cm hexagonal chambers constructed of clear Plexiglas sides, stainless steel rod floors, and removable wood tops. Two photocells

beams were located on the side walls of each chamber at a height of 3.0 cm above the floor and were used to measure horizontal locomotor activity. Interuption of a photocell beam was recorded by a computer.

The 18 μm thick semi-impermeable U-shaped dialysis membrane (Spectra/Por; Spectrum Medical) had an inside diameter of 150 μm was impermeable to molecules with a molecular weight greater than 6000. The concentrations of DA, DOPAC, HVA, and 5-HIAA were quantified using high performance liquid chromatography (Waters 510 HPLC Pump; Millipore) with reverse-phase columns (10 x 0.46 cm Spherisorb-ODS2, 3 μm; Chromatography Sciences Co.) and electrochemical detection (Coulochem Model 5100A, ESA; Conditioning cell Model 5021; Analytical cell Model 5011). The mobile phase contained 0.076 M SDS, 0.1 M EDTA, 15% acetonitrile, 0.058 M sodium phosphate-monobasic pH adjusted to 4.0 with citric acid. Monoamine peak heights were quantified by Waters 746 and 740 Data Modules (Millipore).

*Surgery*

For drug infusions, 21 mm long, 22 gauge guide cannulae were aimed, bilaterally, 1.0 mm above the VTA at the following coordinates according to the atlas of Paxinos and Watson (1986) : - 5.7 caudal to bregma, + 0.6 mm lateral from midline, and - 7.2 ventral from the dura mater. The stereotaxic arms were angled at 15 degrees and the skull was leveled between lambda and bregma (i.e. flat skull position).

A second guide cannula (21 mm long, 20 gauge) for the vertical dialysis probe (Robinson & Whishaw, 1988), was implanted, unilaterally, in the NAS on the right side of the brain at the following coordinates : + 3.0 mm rostral from bregma, + 1.4 mm lateral from midline, and - 6.3 mm ventral from the skull surface (Pellegrino *et al.*, 1979). The cannula was lowered at a lateral angle of 10 degrees and the incisor bar was set 5.0 mm above the interaural line. Following surgery, a stainless steel obturator was inserted into the guide cannula and was flush with its tip.

*Drugs*

DiMe-C7 was used exactly as described in Experiment 1.

*Design and Procedure*

Seven days following surgery, animals were transported to the test room where a dialysis probe was inserted in the NAS. The dialysis probes extended 2.0 mm beyond the tip of the guide cannulae. Animals were then placed in the test chambers and left undisturbed overnight until testing began 17 hours later. During this habituation period, the probe was perfused at 0.1 µl/min with Ringer solution (145.0 mM $Na^+$, 0.2 mM ascorbate, 2.7 mM $K^+$, 1.2 mM $Ca^{++}$, 1.0 mM $Mg^{++}$, 150.0 mM $Cl^-$, 2.0 mM $Na_2HPO_4$; pH = 7.4 ± 0.1). In addition, animals had free access to food and water and were maintained in reverse lighting cycle. Testing began between 09:00 and 10:00. At this time, food was removed but water remained, the dialysate flow rate was increased to 1.5 µl/min, and serial 20 minute samples were taken. When a stable baseline was established, animals were injected with formalin into one hind paw. Twenty minutes later, immediately following the first post-baseline sample collection, animals received bilateral intra-VTA infusions of DiMe-C7 (3.0 µg/0.5 µl/side), or the vehicle, in a counterbalanced between-subjects design. Collected samples (approximately 30 µl) were immediately injected in a volume of 25 µl in the HPLC for content analysis. Animals were tested in an illuminated room during the dark portion of the light-dark cycle.

*Statistical analyses*

Raw data from each dependent variable were analyzed by two-way ANOVAs with Treatment (DiMe-C7 vs vehicle) as the between-subjects variable and Time (blocks of 20 minutes) as the within-subjects variable. There were eleven levels of the Time variable and these included the last five preinjection baseline and six post-baseline time points. These analyses were followed, if appropriate, by simple main effects tests. The neurochemical data presented in figures were transformed to percentages of the mean of the six preinjection baseline samples.

## Results

Figure 5 A illustrates the time course of extracellular DA of animals given intra-VTA DiMe-C7, or the vehicle, 20 minutes following a formalin injection. During post-baseline, DiMe-C7 caused large increases in extracellular DA relative to the vehicle, as evidenced by a significant Treatment x Time interaction, $F(10, 90) = 3.88$, $p < 0.001$. Simple main effects tests indicated that the differences between DiMe-C7 and vehicle were significant at all times, except at 20 minutes, following the intracranial infusions.

Similar findings were observed with the DA metabolite, DOPAC. Figure 5 B shows that intra-VTA DiMe-C7 caused potent increases in extracellular DOPAC as compared to intra-VTA vehicle. There was a significant main effect of Treatment, $F(1,9) = 6.2$, $p < 0.05$, and a significant Treatment x Time interaction, $F(10,90) = 11.7$, $p < 0.0001$. Simple main effects tests revealed that the differences between DiMe-C7 and vehicle were significant at all times, except at 20 minutes, following the intracranial infusions.

As for HVA (Figure 5 C), another DA metabolite, DiMe-C7-treated animals displayed increases in extracellular HVA relative to vehicle-treated animals, but these changes were only marginally significant, $F(10, 80) = 1.94$, $p = 0.0512$.

The effect of intra-VTA DiMe-C7 or the vehicle on extracellular NAS levels of the serotonin metabolite, 5-HIAA, is illustrated in Figure 6 A. There were no differences in extracellular 5-HIAA between intra-VTA DiMe-C7 and the vehicle, as reflected by a non-significant main effect of Treatment, $F(1,9) = 0.05$, $p = 0.83$.

The mean basal levels of neurotransmitter and metabolites in the NAS were : DA : $1.46 \pm 0.08$ pg/25 µl; DOPAC : $1420.2 \pm 63.6$ pg/25 µl; HVA : $1889.1 \pm 107.07$ pg/25 µl; 5-HIAA : $206.7 \pm 7.3$ pg/25 µl.

Figure 6 B illustrates the effect of intra-VTA DiMe-C7 or the vehicle infused 20 minutes following a formalin injection on locomotor activity. As shown, intra-VTA DiMe-C7 stimulated locomotor activity relative to the vehicle. There was a significant

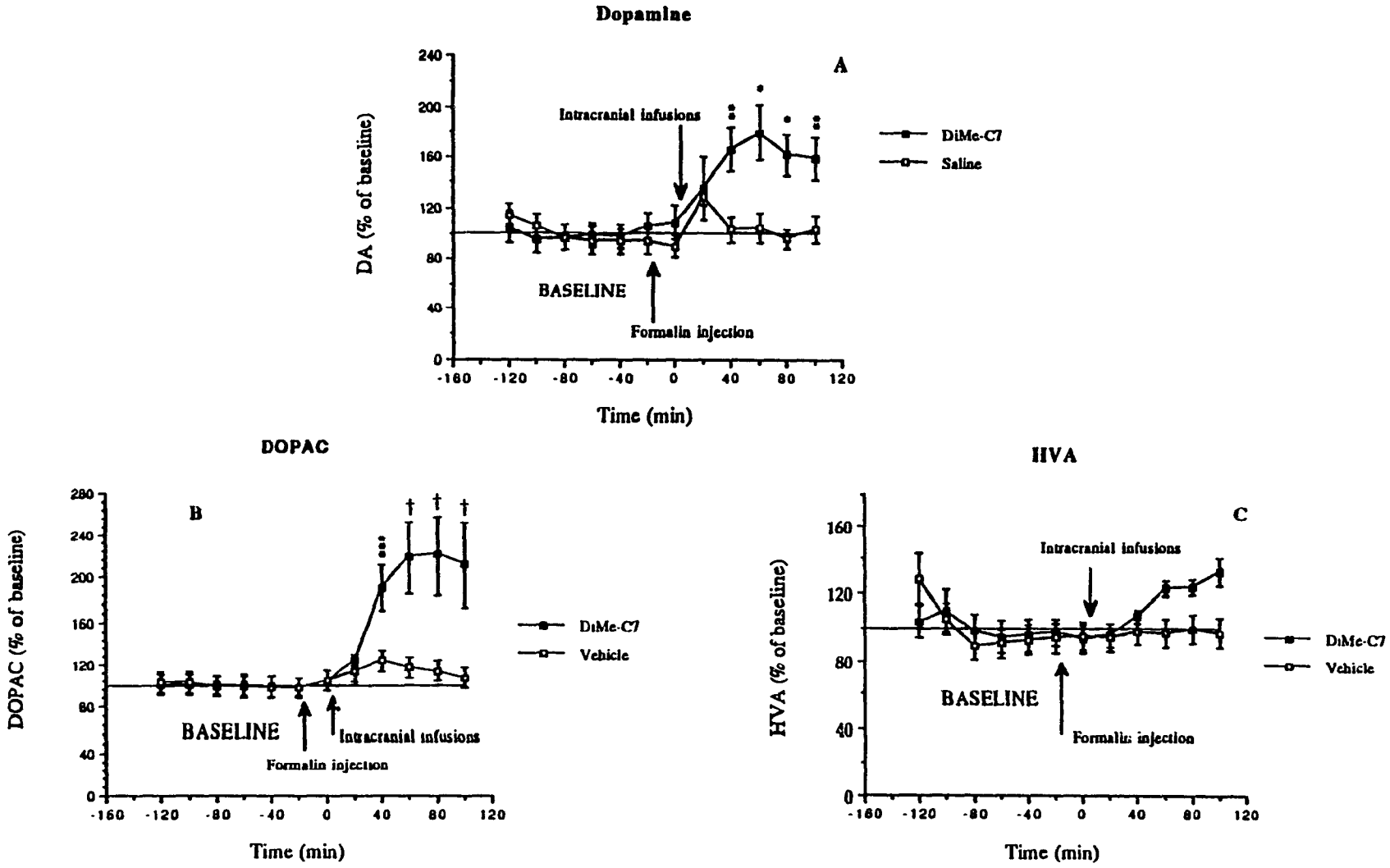


Figure 5. Effect of intra-VTA DiMe-C7 (3.0 µg/0.5 µl/side), or the vehicle, infused 20 minutes following a formalin injection on extracellular NAS levels of (A) DA, (B) DOPAC, and (C) HVA. Rats (n=11) were tested in a between-subjects design. Significant differences between DiMe-C7 and vehicle: * $p < 0.05$; ** $p < 0.025$; *** $p < 0.005$; † $p < 0.001$

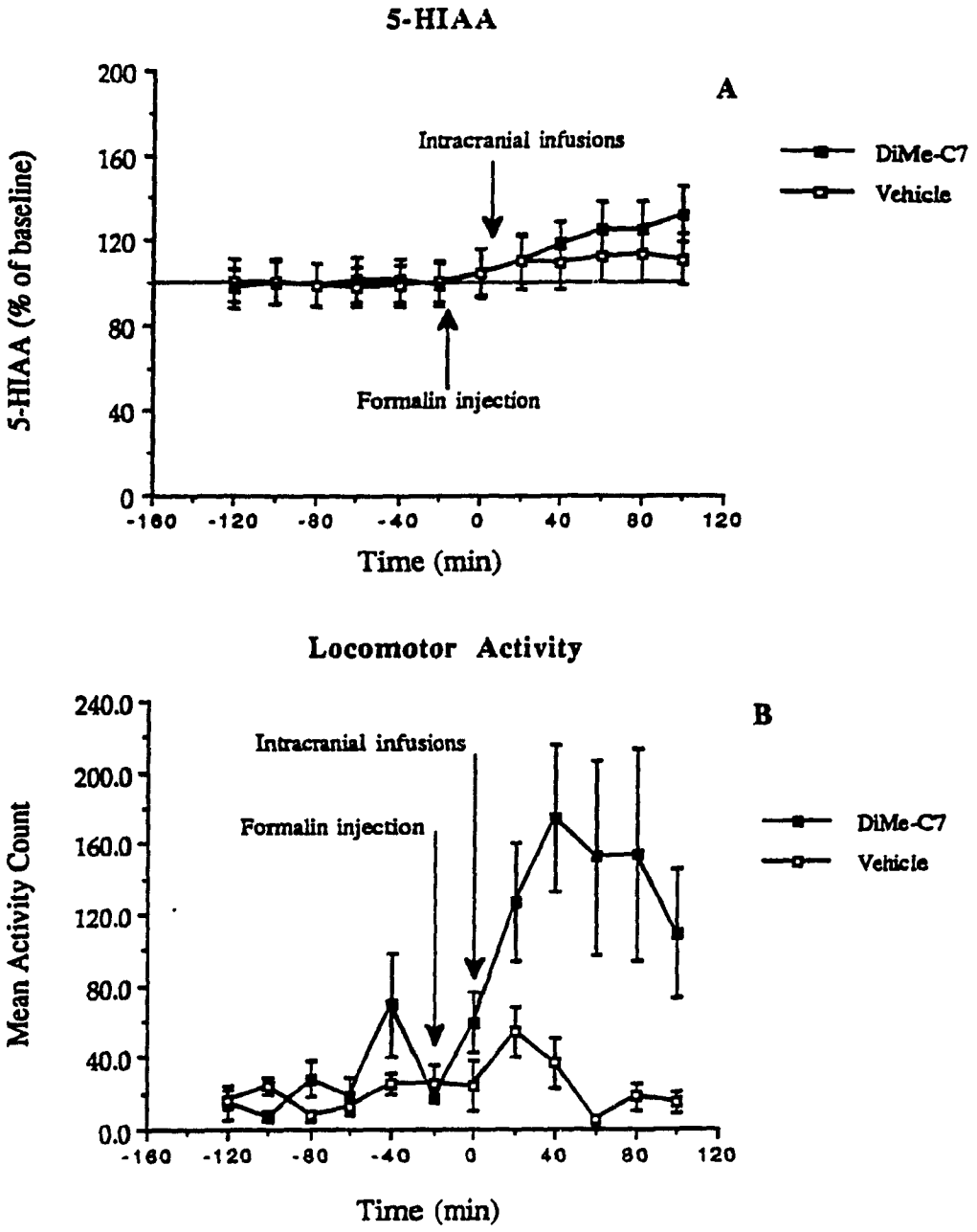


Figure 6. Effect of intra-VTA DiMe-C7 (3.0 μg/0.5 μl/side), or the vehicle, infused 20 minutes following a formalin injection on (A) extracellular NAS levels of 5-HIAA, and (B) on locomotor activity. Rats (n=11) were tested in a between-subjects design. Significant differences between DiMe-C7 and vehicle : ** $p < 0.01$; † $p < 0.001$.

main effect of Treatment, $F(1,9) = 15.2$, $p < 0.004$, and a significant Treatment x Time interaction, $F(10, 90) = 5.18$, $p < 0.0001$. Simple main effects tests indicated that the differences between DiMe-C7 and vehicle were significant at all times following the intracranial infusions.

The location of the internal injector cannulae and probe tips of rats tested in this experiment is illustrated in Figure 7 (for the VTA) and Figure 8 (for the NAS). As shown in these figures, 11 rats had their internal injector cannulae tips within the VTA and the probe tip within the NAS. The data for one rat were discarded because its probe tip was not within the NAS.

## Discussion

The results indicate that intra-VTA DiMe-C7 infused just prior to the late pain phase of the formalin test causes large elevations in extracellular NAS levels of DA and its metabolite, DOPAC, suggesting enhanced DA release (Imperato & Di Chiara, 1984) in the NAS. These biochemical changes were observed 40 minutes, and persisted during at least 100 minutes, following the intracranial infusions. These results are consistent with those of previous studies showing that intra-VTA (Cador *et al.*, 1989; Elliott *et al.*, 1986) and peripheral (Boix *et al.*, 1992a,b) administration of SP or DiMe-C7 increases indices of DA activity in the NAS.

Intra-VTA DiMe-C7 also stimulated locomotor activity, as had been observed informally in Experiment 1. This finding confirms that of previous studies (Eison *et al.*, 1982a,b), although the locomotor stimulant effect of DiMe-C7 was much more prolonged in the present study, lasting for at least an additional 50-60 minutes. This difference in the time-course of the effect of DiMe-C7 was probably due to the higher dose used in the present study. In the Eison et al (1982a) study, for instance, DiMe-C7 was infused at a dose of 2.5 µg/rat, whereas in the present study, a dose of 6.0 µg/rat was used.

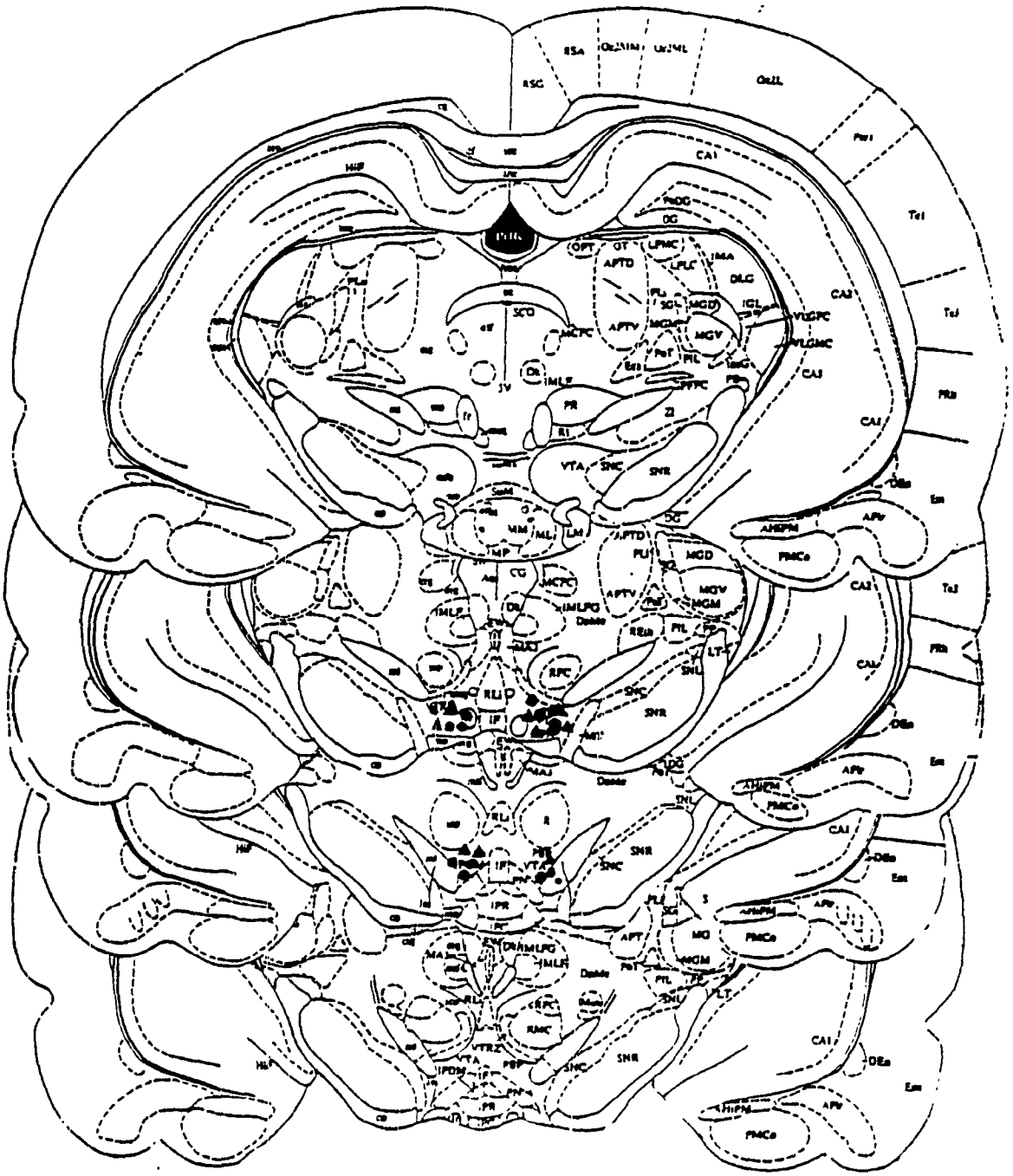

Figure 7. Location of the internal injector cannulae tips in the VTA of rats t ested in Experiment 2 that received DiMe-C7 (circles), or the vehicle (triangles). Drawings are from the atlas by Paxinos and Watson (1986).

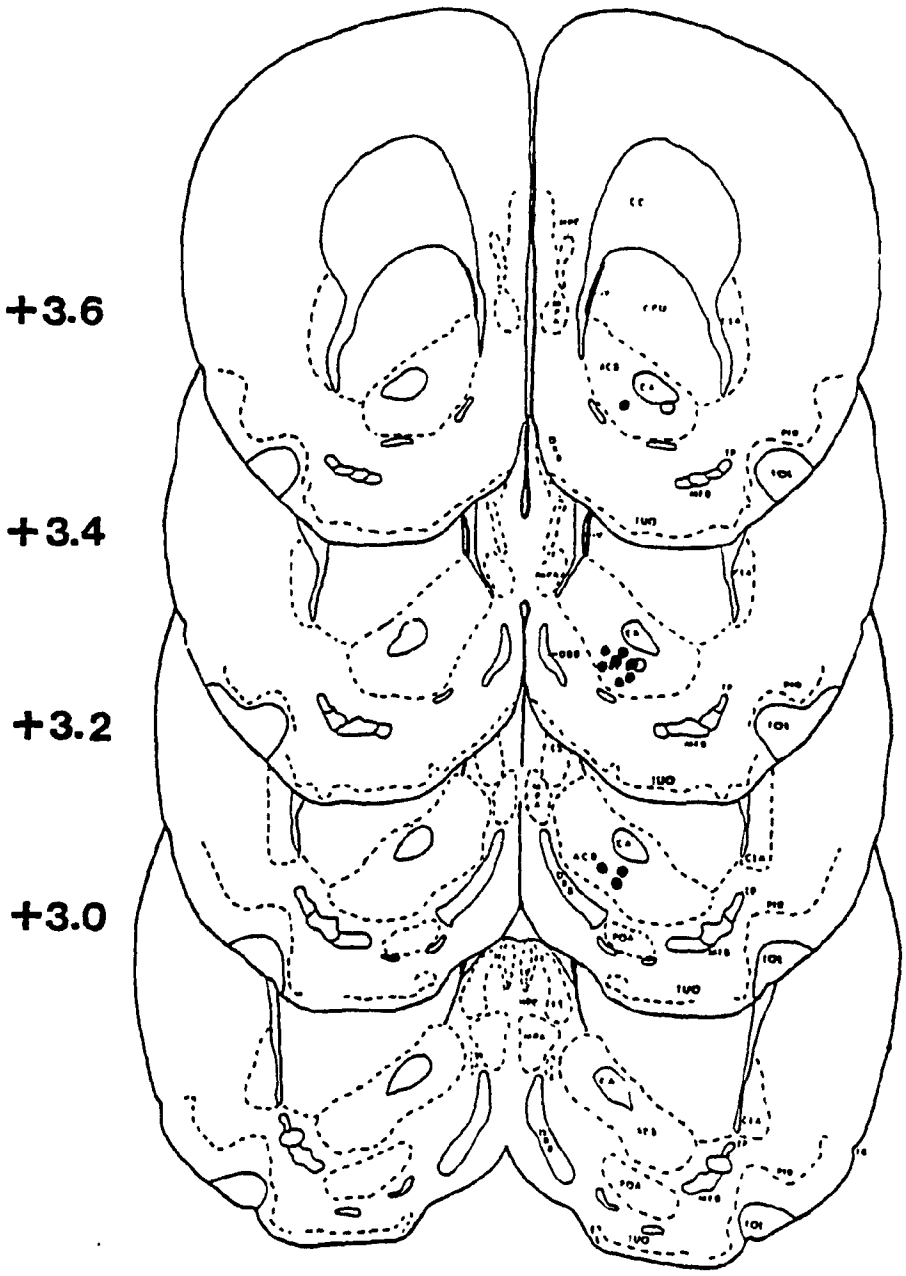


Figure 8. Location of the probe tips in the NAS of rats tested in Experiment 2. Drawings are from the atlas by Pellegrino *et al.* (1979).

The findings that intra-VTA DiMe-C7 infused prior to the late tonic pain phase produces analgesia, as observed in Experiment 1, and increases NAS levels of DA and its metabolites suggest that the SP analogue induces analgesia in the formalin test by causing DA release from terminals of mesolimbic neurons innervating the NAS. There is a temporal discrepancy, however, between the analgesic effect of intra-VTA DiMe-C7 and the increase in apparent DA release in the NAS that contradicts the latter proposal. More specifically, DiMe-C7 increased extracellular levels of DA and its metabolites during at least 100 minutes and yet the analgesia produced by theSP analogue lasted for only 39 minutes. This temporal discrepancy raises the prospect that DiMe-C7 produces analgesia, not by enhancing DA release in the NAS, but rather by causing the biochemical activation of DA neurons innervating different terminal fields, such as the amygdala or the prefrontal cortex. Still, it is possible that non-dopaminergic mechanisms function to underlie DiMe-C7-induced analgesia. For instance, DiMe-C7 might interact with serotonergic systems given the evidence that serotonin plays a role in stress-induced analgesia in the formalin test (Abbott *et al.*, 1986), median raphe projections course through the VTA (Bobillier *et al.*, 1979), and median raphe lesions affect morphine-induced analgesia in the formalin test (Abbott & Melzack, 1982). It remains to be determined, however, whether DiMe-C7 has any affinity for serotonin receptors (e.g. 5-HT1 and 5-HT2).

Intra-VTA DiMe-C7 was also observed to stimulate hind limb scratching and wet dog shakes. These behavioral effects were reported previously following intra-VTA (Eison *et al.*, 1982a) and intracerebroventricular (Elliott & Iversen, 1986) infusions of DiMe-C7. Eison et al (1982a) found that these stimulant effects are specifically induced by DiMe-C7, as opposed to SP, and suggested that these differences could be due to either DiMe-C7's more prolonged pharmacological action or to the activation of distinct neural structures by this peptide.

In summary, DiMe-C7 infused in the VTA just prior to the late pain phase of the formalin test activates DA neurotransmission in mesolimbic neurons projecting to the NAS, as evidenced by increased levels of extracellular DA and DOPAC and, to a lesser extent, HVA in the NAS. These results suggest that intra-VTA DiMe-C7 might produce analgesia in the formalin test by enhancing DA release in the NAS.

## EXPERIMENT 3

## Effects of Intra-NAS and Intra-mPFC Infusions of Amphetamine in the Formalin Test

The findings that intra-VTA infusions of a SP analogue inhibit formalin pain responses and cause DA release in the NAS suggest that activation of mesolimbic DA neurons projecting to the NAS mediates the analgesic effects of intra-VTA DiMe-C7. As previously mentioned, there is reason to believe that DA release from terminals in the mPFC may also underlie DiMe-C7-induced analgesia. As a follow-up to Experiment 2, the following experiment examined the differential effects of activating midbrain DA terminal fields, presumed to underlie DiMe-C7-induced analgesia, on pain responses in the formalin test. To this end, the effects of amphetamine-induced DA release in the mPFC and in the NAS in this test were studied.

### Method

*Apparatus*

Apparatus were identical to those used in Experiment 1.

*Surgery*

For the NAS, 19 mm long, 22 gauge guide cannulae were implanted bilaterally 1.0 mm above this site at the following coordinates: + 3.0 mm rostral from bregma, + 1.4 mm lateral from midline, and - 6.3 mm ventral from the skull surface (Pellegrino *et al.*, 1979).

Cannulae were lowered at a lateral angle of 10 degrees and the incisor bar was set 5.0 mm above the interaural line.

In the case of the mPFC, 16 mm long, 22 gauge guide cannulae were implanted bilaterally at the following coordinates:+ 3.6 mm rostral from bregma, + 0.4 mm lateral from midline, and -2.4 mm ventral from the dura mater (Paxinos & Watson, 1986). The stereotaxic arms were angled at 15 degrees and the skull was leveled between lambda and bregma (i.e. flat skull position). While removing the skull extending from above both mPFC placements, extreme precaution was taken to avoid puncturing the midsaggital sinus.

*Drugs*

D-amphetamine sulfate (Smith Kline Beecham, Oakville, Ont) was used as a tool to cause DA release and reuptake blockade from presynaptic nerve terminals (Kuzcenski, 1983). It was dissolved in saline and infused bilaterally at either 1.5 or 2.5 µg/0.5µl/side. These amounts of amphetamine were used because they were reported previously to be effective at stimulating DA-dependent locomotor activity (Vezina *et al.*, 1991).

*Design and procedure*

On the test day, rats received bilateral infusions of either 1.5 or 2.5 µg/0.5 µl/side of amphetamine, or the vehicle, using a counterbalanced within-subjects design, into the mPFC or the NAS immediately prior to the formalin injection. Pain responses were continuously recorded for 75-minutes.

*Statistical Analyses*

Data were analyzed by two-way ANOVAs with Treatment (combined amphetamine doses vs vehicle) and Time (25 blocks of 3 minutes) as within-subjects variables. Data from the two amphetamine doses were combined and compared to vehicle conditions because there were no significant differences in pain responses between these doses when infused into either the mPFC, $F(24,312) = 0.537$, $p = 0.96$, or the NAS, $F(24,312) = 0.541$, $p = 0.9$.

## Results

Figure 9 illustrates the time-course of formalin-induced pain responses immediately following intra-mPFC infusions of amphetamine or the vehicle. Amphetamine was microinfused into this site at doses of either 1.5 or 2.5 μg/0.5 μl/side. Intra-mPFC amphetamine, at either dose tested, failed to alter formalin pain responses. There was no significant Treatment x Time interaction, $F(24,312) = 0.68$, $p = 0.9$. The location of the internal injector cannulae tips is illustrated in Figure 10. Fourteen rats had their injector tips within the mPFC. One rat had its injector tips outside the limits of the mPFC and thus its data were discarded.

Figure 11 shows quite a different effect when amphetamine was infused into the NAS. Both doses of amphetamine greatly attenuated pain responses relative to vehicle, as evidenced by a significant Treatment x Time interaction, $F(24,336) = 3.01$, $p < 0.0001$. Tests for simple main effects revealed that the differences between amphetamine and the vehicle were significant at all time points during the first 24 minutes, except at 3 minutes, following the formalin injection. Figure 12 illustrates the location of the internal injector cannulae tips. Fifteen rats had their injector tips within the NAS. One rat failed to satisfy the criterion for inclusion and thus its data were discarded.

## Discussion

It has been reported previously that peripheral administration of amphetamine can induce analgesia in the formalin test (Clarke & Franklin, 1992; Morgan & Franklin, 1990; Skaburskis, 1980). In the present experiment, amphetamine was found to produce analgesia in this test when infused directly into the NAS. This finding suggests that the analgesia produced by peripheral administration of amphetamine in the formalin test is due, at least in part, to amphetamine-induced release of DA in the NAS. The present results confirm those of Morgan (1990) who found that intra-NAS infusions of

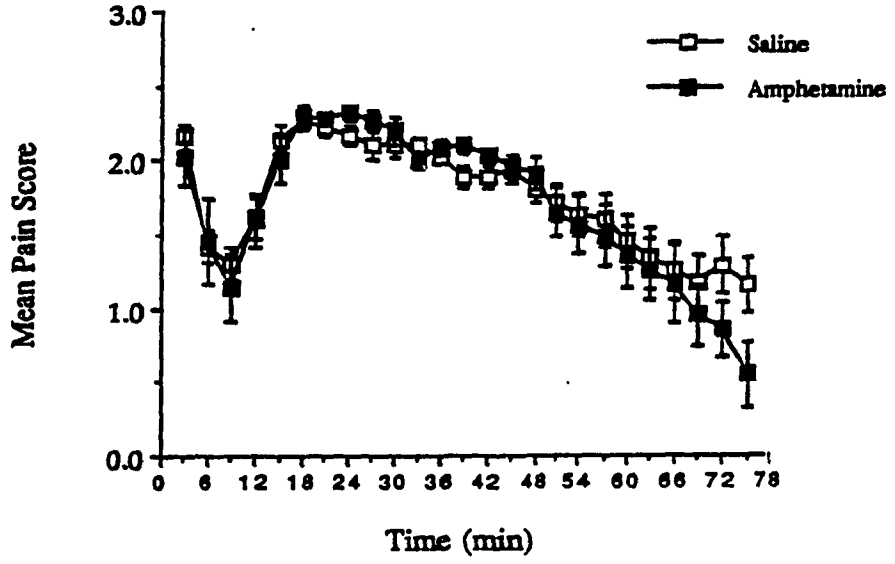


Figure 9. Time course of mean formalin pain scores (± S.E.M) immediately following bilateral intra-mPFC infusions of amphetamine (combined 1.5 and 2.5 μg/0.5 μl/side) or saline.

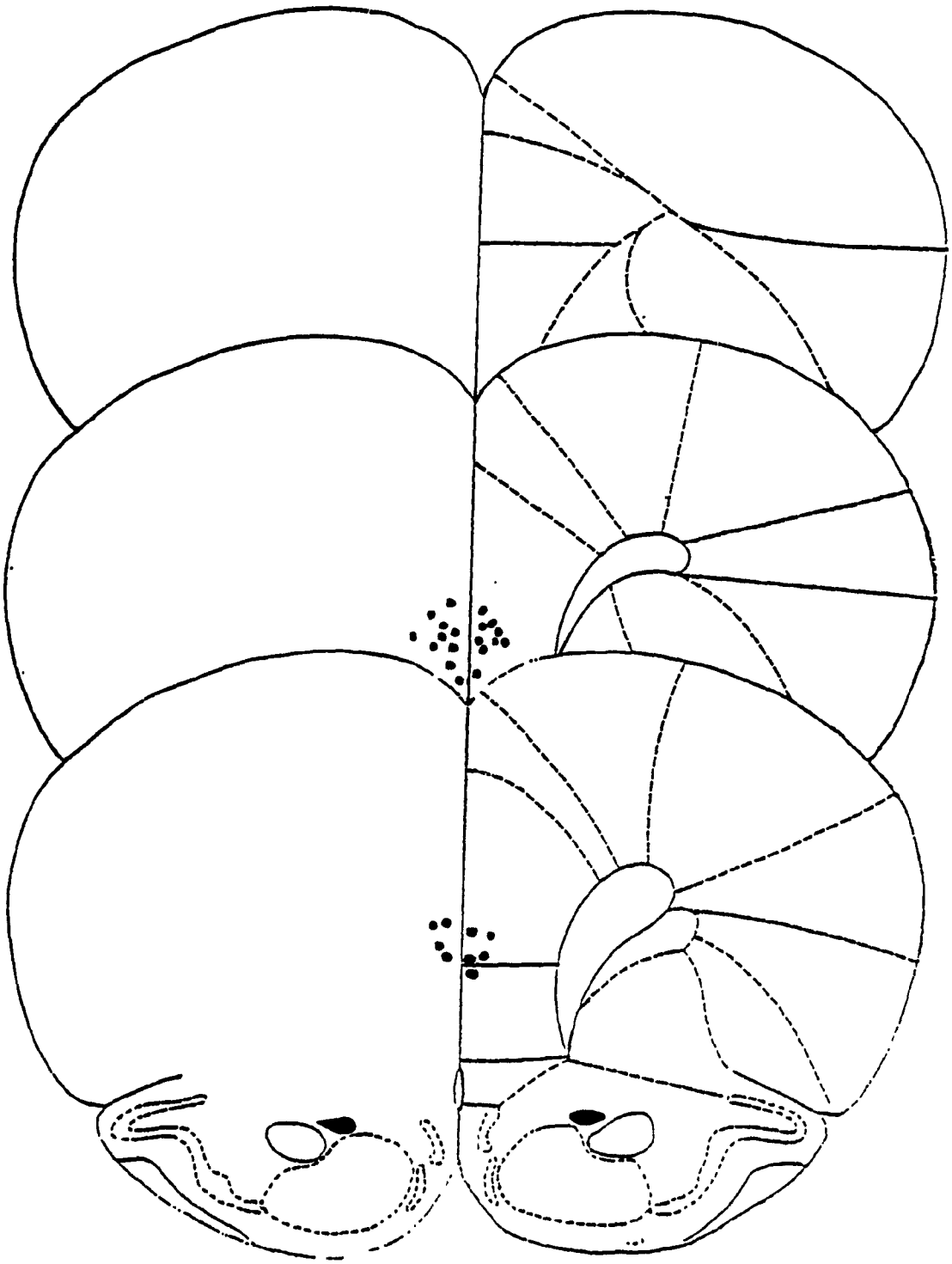

Figure 10. Location of the internal injector cannulae tips in the mPFC of rats that received microinfusions of amphetamine immediately prior to a formalin injection. Drawings are from the atlas by Paxinos and Watson (1986).

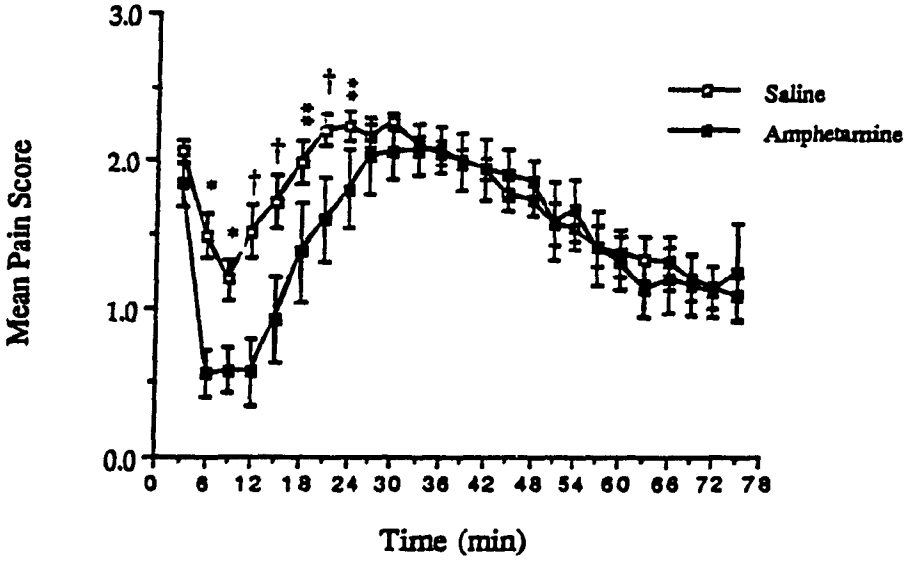


Figure 11. Effect of bilateral intra-NAS infusions of amphetamine (combined 1.5 and 2.5 µg/0.5 µl/side), or saline, in the formalin test. Differences between amphetamine (combined doses) and vehicle mean pain scores were significant at all time points during the first 24 minutes, except at 3 minutes, following the formalin injection (** $p < 0.05$; * $p < 0.025$; † $p < 0.01$).

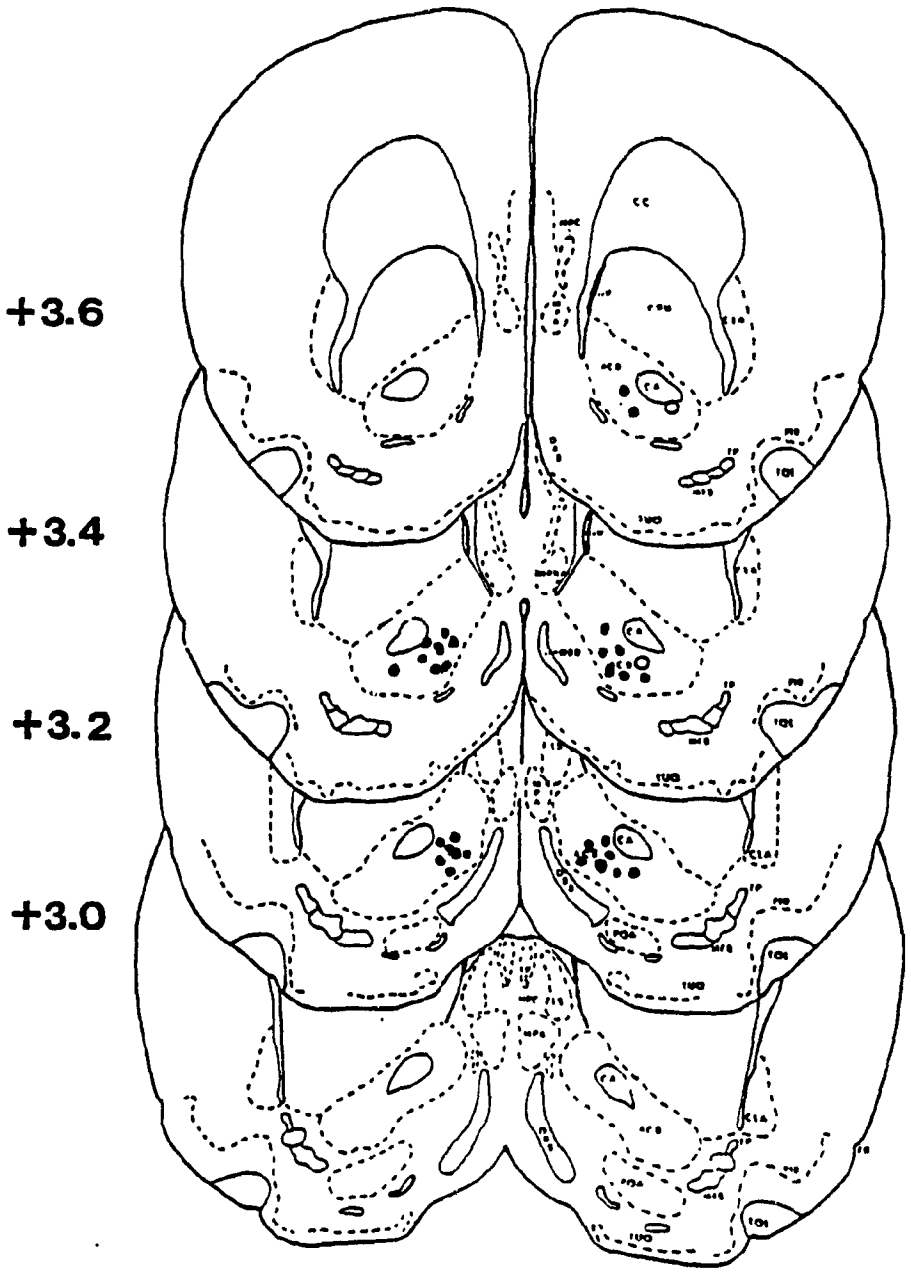


Figure 12. Location of the internal injector cannulae tips in the NAS of rats that received microinfusions of amphetamine immediately prior to a formalin injection. Drawings are from the atlas by Pellegrino *et al.* (1979).

amphetamine, using 10.0 and 20.0 μg/rat, produces dose-dependent analgesia in the formalin test .

One somewhat surprising result from these experiments was that amphetamine suppressed pain in the formalin test when infused into the NAS, but not when infused into the mPFC. As previously mentioned, there is evidence that in response to mild stressors there is preferential activation of mPFC over NAS DA projections (Thierry *et al.*, 1976), and that this activation is SP dependent (Bannon *et al.*, 1983). It was expected, therefore, that amphetamine into this region might inhibit pain responses in the formalin test.

There appears to be some analogy between the present findings on analgesia and those of a previous study (Vezina *et al.*, 1991) on the effects of intra-NAS and mPFC amphetamine-induced DA release on locomotor activity. Amphetamine, when infused into the NAS at both 1.5 and 2.5 μg/0.5 μl/side doses of amphetamine stimulated locomotor activity whereas, when infused into the mPFC, neither dose of amphetamine altered locomotor activity. These findings suggest that analgesia in the formalin pain test and locomotor activity are mediated by a common neurocircuitry.

It has been suggested that DA release in the mPFC can inhibit locomotor activity by modulating DAergic neurotransmission in subcortical regions such as the NAS. For instance, DA-depleting 6-hydroxydopamine lesions of the mPFC enhance amphetamine-induced activity (Carter & Pycock, 1980) and enhance subcortical DA utilization in the NAS (Pycock *et al.*, 1980 a, b). More recently, direct evidence for the inhibitory role of mPFC DA on amphetamine-induced locomotor activity was provided by the finding that amphetamine-induced DA release in the mPFC could inhibit the locomotor-activating effects of intra-NAS amphetamine infusions (Vezina *et al.*, 1991). Given the parallels between the effects of intra-NAS and mPFC amphetamine on pain responses in the formalin test and locomotor activity, it is possible that DA release in the mPFC inhibits analgesia by modulating DA activity in the NAS.

In summary, the results show that intra-NAS, but not intra-mPFC, amphetamine-induced DA release causes analgesia in the formalin test. One of the most striking features of these data is that the pattern of analgesia induced by intra-NAS amphetamine resembles that induced by intra-VTA DiMe-C7. Pain responses were attenuated to a similar extent during approximately 25 minutes following the formalin injection. Thus, it would appear that intra-VTA DiMe-C7 produces analgesia by activating DA neurons projecting to the NAS. The findings from Experiment 2 that intra-VTA DiMe-C7 infused during the course of formalin testing increased extracellular DA into the NAS lend support to this idea.

## EXPERIMENT 4

### Effects of Intra-VTA DiMe-C7 in the Tail-Flick Test

Previous work has shown that the neural systems mediating analgesia in the formalin pain test are fundamentally different from those in phasic tests, such as the spinal reflex withdrawal tail-flick test (e.g. Abbott *et al.*, 1981; Abbott & Melzack, 1982, 1983). For example, Morgan and Franklin (1990) showed that lesions of the VTA block the analgesic effects of morphine and amphetamine in the formalin but not the tail-flick test, suggesting that midbrain DA systems mediate drug-induced analgesia in the former but not the latter test. Given these findings, it was of interest to compare the effects of intra-VTA DiMe-C7, and in Experiment 5 intra-mPFC and NAS amphetamine, in the formalin test to those in the tail-flick test.

## Method

### *Subjects*

All animals tested in Experiment 1 (except for those receiving DiMe-C7 25 minutes following a formalin injection) were assessed, two weeks later, in the tail-flick test.

### *Apparatus*

The tail-flick test apparatus consisted of a wood platform mounted on the rim of a 43 x 30 x 22 cm clear Plexiglas tank. The tank water was heated at 55 °C by a Haake E2 Immersion/Open Bath Circulator. Between tests, animals were kept in clear Plexiglas shoebox cages with wire tops.

### *Drugs*

DiMe-C7 was used exactly as described in Experiment 1.

### *Design and Procedure*

On the test day, rats received bilateral intra-VTA infusions of DiMe-C7 (3.0 µg/0.5 µl/side), or the vehicle, in a counterbalanced within-subjects design and tail-flick tests trials were administered immediately once every 10 minutes for 1 hour.

### *Statistical Analyses*

Data were analyzed by a two-way ANOVA with repeated-measures on both Treatment (DiMe-C7 vs vehicle) and Time (7 test blocks) variables.

## Results

The effect of intra-VTA infusions of DiMe-C7, or the vehicle, on tail-flick latencies is illustrated in Figure 13 A. DiMe-C7 decreased mean tail-flick latencies

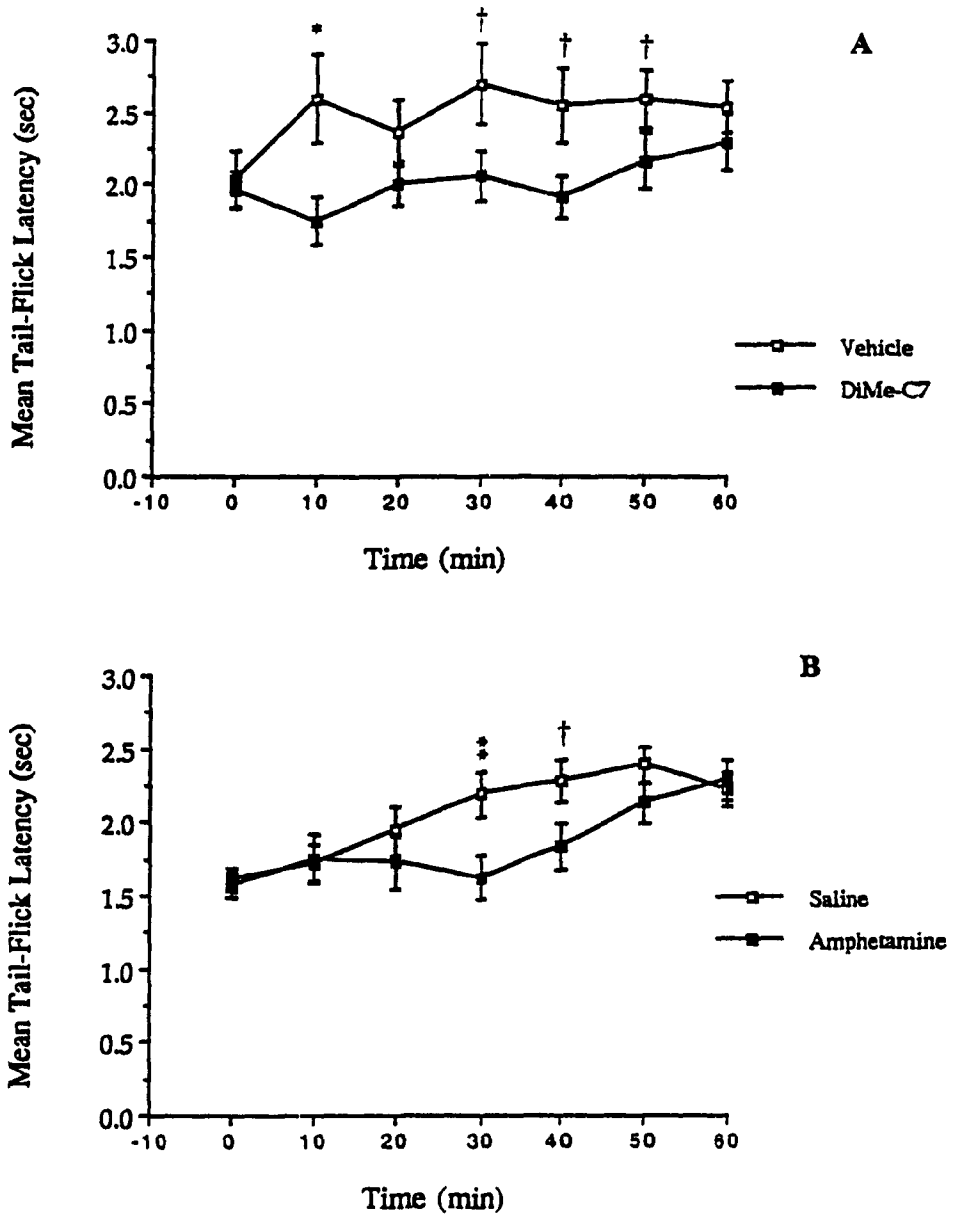


Figure 13. Effect of bilateral (A) intra-VTA infusions of DiMe-C7 (3.0 µg/0.5 µl/side; n=12 ), or the vehicle, and (B) intra-NAS amphetamine (1.5 and 2.5 µg/0.5 µl/side; n=14) on mean tail-flick latencies (± S.E.M.). Rats were administered the intracranial infusions in a counterbalanced within-subjects design and tests were begun immediately, once every 10 minutes for 1 hour. Significantly different from vehicle : † $p < 0.05$; * $p < 0.025$; ** $p < 0.005$.

relative to the vehicle, suggesting, if anything, that the neuropeptide increased pain sensitivity. There was a significant main effect of Treatment, $F(1,11) = 6.73$, $p < 0.05$.

## EXPERIMENT 5

## Effect of Intra-NAS and Intra-mPFC Amphetamine in the Tail-Flick Test

### Method

*Subjects*

All rats used in Experiment 3 were assessed, two weeks later, in the tail-flick test.

*Apparatus*

Apparatus were identical to those used in Experiment 4.

*Drugs*

D-amphetamine was used exactly as described in Experiment 3.

*Design and procedure*

On the test day, rats were administered tail-flick test trials immediately following bilateral infusions of amphetamine (1.5 or 2.5 µg/0.5 µl/side), or the vehicle, using a counterbalanced within-subjects design, into either the NAS or the mPFC.

*Statistical Analyses*

Data were analyzed by two-way ANOVAs with Treatment (amphetamine vs vehicle) and Time (7 test blocks) as within-subjects variables. For the NAS, data from both amphetamine doses were combined and compared to the vehicle condition because there were no significant differences in tail-flick latencies between these doses, $F(1,12) = 0.04$, $p = 0.9$. In the case of the mPFC, separate ANOVAs were conducted on each drug dose (i.e. 3.0 µg vs saline and 5.0 µg vs saline) because there were significant differences in tail-flick latencies between these doses, $F(1,13) = 5.06$, $p < 0.05$.

## Results

As shown in Figure 13 B, when amphetamine was infused, at either dose, into the NAS, there was a tendency for decreased tail-flick latencies relative to the vehicle. This observation was supported by a significant Treatment x Time interaction, $F(6,78) = 2.9$, $p < 0.05$. Simple main effects tests revealed that the differences in tail-flick latencies between amphetamine and vehicle were significant at 30 and 40 minutes, indicating that the DA agonist caused hyperalgesia at these time points.

Figure 14 A illustrates the effect of amphetamine infused in the mPFC at a dose of 3 μg/1 μl in the tail-flick test. As shown, intra-mPFC amphetamine was without effect on tail-flick latencies. The Treatment x Time interaction was not significant, $F(6,36) = 2.03$, $p = 0.09$. Inspection of Figure 14 A indicates that amphetamine increased tail-flick latencies at 30 and 60 minutes.

As shown in Figure 14 B, when amphetamine was infused in the mPFC at a dose of 5 μg/1 μl, it had no effect on tail-flick latencies, except at 40 minutes where it appeared to elevate latencies. The Treatment x Time interaction was not significant, $F(6,42) = 1.77$, $p = 0.13$.

## Discussion

Intra-VTA DiMe-C7 and intra-NAS infusions of amphetamine did not induce analgesia as measured in the tail-flick test. These results parallel those of a previous study (Morgan and Franklin, 1990), indicating that midbrain DA systems play a role in analgesia in the formalin, but not in the tail-flick pain test.

The results obtained from the present experiments provide direct evidence that activation of midbrain DA systems by DiMe-C7 applied to the VTA and amphetamine applied to the NAS produce different effects in these two pain tests. In the course of investigating the effect of identical manipulations of midbrain DA systems in these two pain tests, we have also observed (unpublished results) that intra-VTA infusions of

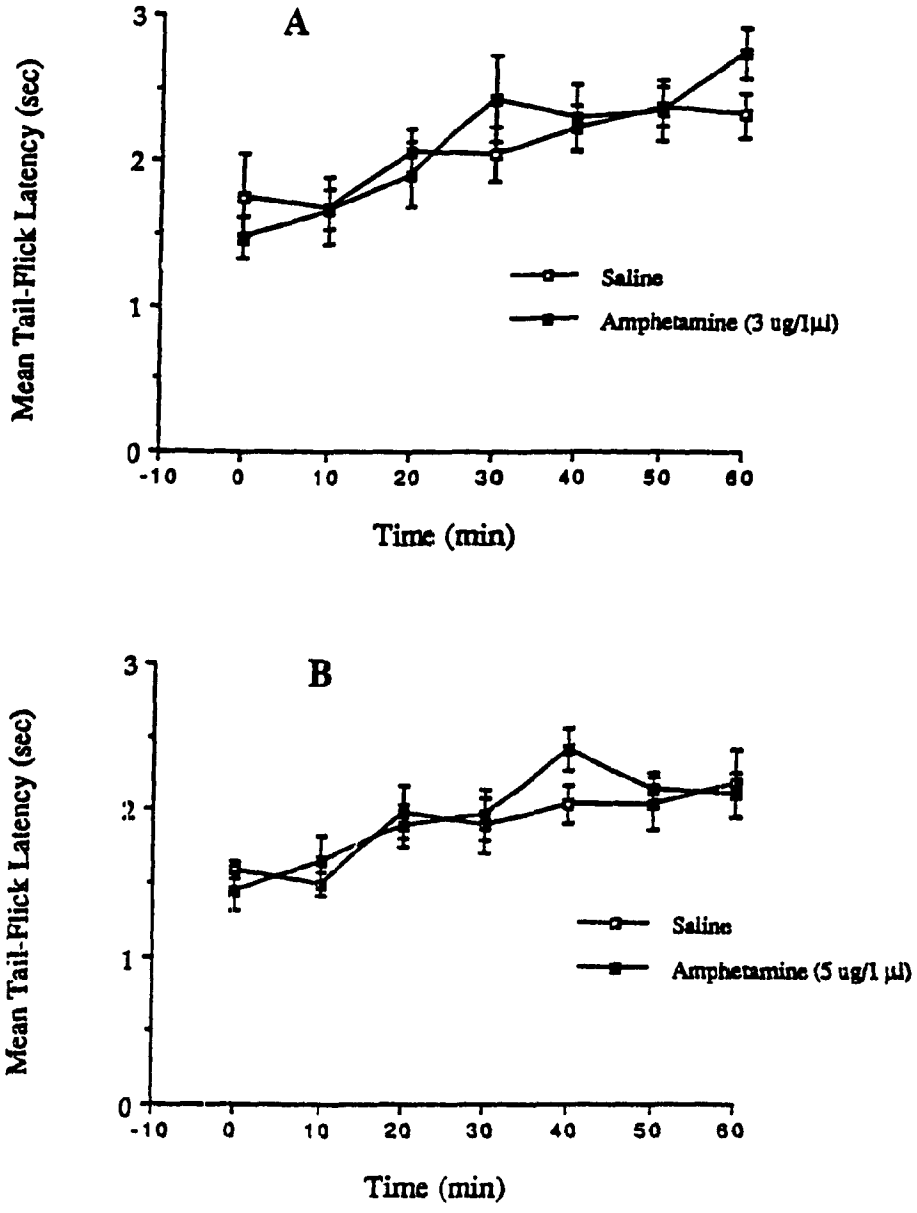


Figure 14. Effect of amphetamine infused into the mPFC at a dose of either (A) 1.5 µg/0.5 µl/side; (n=7), or (B) 2.5 µg/0.5 µl/side (n=8), on mean tail-flick latencies (± S.E.M.).

morphine, at a dose (3.0 μg/0.5 μl/side) previously reported to produce analgesia in the formalin test (Morgan, 1990), had no effect on nociceptive thresholds in the tail-flick test. Similar findings have been reported by Moreau *et al.* (1985). Together, these findings reinforce the idea that a distinction with respect to the neural substrates of analgesia should be made between these tonic and phasic pain tests.

Another finding of interest is that intra-VTA DiMe-C7 caused a hyperalgesic response in the tail-flick test, indicating that SP release in the VTA may play a role in the modulation of phasic pain. This effect appeared to be specific to SP, since intra-VTA infusions of morphine (unpublished observations) had no effect on nociceptive thresholds in the tail-flick test. It may be that some action of SP in the VTA on descending spinal mechanisms is responsible for the effect. Furthermore, it has been suggested previously that different fragments of the SP molecule exert opposite effects on nociceptive thresholds in phasic pain tests; the C-terminal fragment appears to mediate the nociceptive effects of SP whereas the N-terminal fragment appears to mediate the antinociceptive effects of SP (Skilling *et al.*, 1983; Stewart *et al.*, 1982). Though speculative, it is possible that the hyperalgesic effect seen after intra-VTA DiMe-C7 in the tail-flick test is related to the fact that this compound is a C-terminal fragment of SP. The results from the present study also indicate that intra-NAS, but not intra-mPFC, amphetamine causes hyperalgesia in the tail-flick test. Together with the finding that intra-VTA DiMe-C7 causes hyperalgesia in the tail-flick test, these results suggest that activation of midbrain DA neurons innervating the NAS plays a role in the mediation of hyperalgesia when a phasic pain test is employed.

## EXPERIMENTS 6 AND 7

### The Role of Midbrain SP in Stress-Induced Analgesia

A wide variety of stressors have been shown to inhibit pain responses in the formalin test. Some of these include swimming (Vaccarino *et al.*, 1992 a, b), footshock (Fanselow, 1984; Fanselow & Baackes, 1982; Fanselow *et al.*, 1988; Fanselow *et al.*, 1989; Fanselow & Helmstetter, 1988; Helmstetter, 1992; Helmstetter & Fanselow, 1987; Maier *et al.*, 1984), environmental novelty (Abbott *et al.*, 1986), handling stimuli and odors released by stressed conspecifics (Fanselow & Sigmundi, 1986), and exposure to a predator (Lester & Fanselow, 1985). Interestingly, some of these stressors have been shown to selectively activate DAergic neurotransmission in ascending midbrain neurons (Deutch & Roth, 1990). Other data indicate that SP release in the VTA may play a critical role in the stress-induced activation of midbrain DA systems since, in one study (Bannon et al., 1983), intra-VTA infusions of a SP antibody completely prevented this response.

The evidence that stressors inhibit formalin pain responses and cause SP-induced activation of midbrain DA neurons suggests that SP release in the VTA might underlie stress-induced analgesia in the formalin test. Support for this hypothesis is provided by the findings obtained in Experiment 1 that intra-VTA infusions of a SP analogue induce analgesia in the formalin test. The purpose of the following experiments was to explore the role of midbrain SP on stress-induced analgesia in the formalin test. Because footshock was used as a stressor to demonstrate the role of SP in the stress-induced activation of midbrain DA neurons, the same stressor was employed in the present experiments to induce analgesia. The parameters of footshock-stress used here (three 1-sec, 1-mA shocks at 20-sec intervals) were similar to those used by Fanselow (1984; three 0.75-sec, 1-mA shocks at 20-sec intervals), who found that these parameters cause potent

analgesia in the formalin test. Other procedural details (e.g. time after the formalin injection at which footshock was applied) were as in Fanselow's (1984) study.

## EXPERIMENT 6

## Effects of Intra-VTA Applications of the NK-1 Tachykinin Receptor Antagonist, WIN 51708, on Stress-Induced Analgesia in the Formalin Test

SP, as well as the structurally-related tachykinins, Neurokinin A and B, interact in the peripheral and central nervous systems with at least three receptor subtypes, NK-1, NK-2 and NK-3 (Watling, 1992). SP binds preferentially to NK-1 receptors whereas Neurokinin A and B are the most potent natural ligands of the NK-2 and NK-3 receptors, respectively (Regoli *et al.*, 1983). Although peptide antagonists of the (NK-1) SP receptor have been documented (Snider et al., 1991), several findings suggest that their use has little heuristic value. For instance, their affinity for the SP receptor is low and they are metabolically unstable (Snider *et al.*, 1991). A substantial effort is being devoted to the search for nonpeptide SP receptor antagonists and several discoveries have recently been made (Watling, 1991). Two nonpeptide (NK-1) SP receptor antagonists have been described which bind preferentially to rat brain (Aimone *et al.*, 1991; Appell *et al.*, 1992). One of these, WIN 51708, was used in the present study to explore the role of midbrain SP on stress-induced analgesia in the formalin test.

### Method

*Apparatus*

Footshock stress was conducted in 45 x 26 x 26 cm chambers. The front and rear panels were made of clear Plexiglas, the sides of pressed wood, the top of wire screen, and the floor of 22 stainless steel rods set 1.5 cm apart. Each rod was connected to a shock

generator (Lafayette Instruments, Lafayette, Indiana) . Because these chambers were also used to observe formalin-induced pain responses, a mirror was mounted behind the rear panel to allow a clear view of the rats' paws. Between injections and testing, rats were kept in clear Plexiglas shoebox cages with wire tops.

*Surgery*

21 mm long, 22 gauge guide cannulae were implanted, bilaterally, 1.0 mm above the VTA at the following coordinates : - 5.7 caudal to bregma, + 0.6 mm lateral from midline, and - 7.2 ventral from the dura mater (Paxinos & Watson, 1986). The stereotaxic arms were angled at 15 degrees and the skull was leveled between lambda and bregma (i.e. flat skull position).

*Drugs*

The novel nonpeptide NK-1 tachykinin receptor antagonist, WIN 51708 (Sterling Winthrop Pharmaceuticals) was used. This compound [[1S- (1 alpha, 3A beta, 3b alpha, 5a beta, 15a alpha, 115b beta, 17a alpha)] - 1- ethynyl - 2, 3, 3a, 3b, 4, 5, 5a, 6, 15, 15a, 15b, 16, 17, 17a - tetradecahydro - 15a, 17a - dimethyl - 1H-benzimidazo [2, 1-b]- cyclopenta [b, 6] naptho [1, 2-G]- quinazolin-1-01] (WIN 51708) binds competitively at the NK-1 site in the rat forebrain (Appell *et al.*, 1991). Because of its poor solubilty in saline or acid saline, the compound was applied, bilaterally in the VTA, 30 minutes prior to testing, in crystalline form packed into 28 gauge stainless steel internal injector cannulae.

*Design and procedure*

On the test day, rats received bilateral intra-VTA applications of either WIN 51708 or sham internal injector cannulae followed, 11 minutes later, by a formalin injection into one hind paw. Fifteen minutes following the formalin injection, rats were placed in the footshock chambers and, after a 3-min adaptation period, were exposed to either footshock (three-1 sec, 1-mA shocks at 20-sec intervals) or no footshock. Pain responses were recorded continuously for 55 minutes immediatley following the third shock. Thus, rats

were tested in a counterbalanced between-subjects design and were randomly assigned to one of four groups : footshock + WIN 51708; no footshock + WIN 51708; footshock + sham; no footshock + sham.

*Statistical Analyses*

Data were analyzed by two-way ANOVAs with Treatment (2 levels; see result section for details) as the between-subjects variable and Time (18 blocks of 3 minutes) as the within-subjects variable. These analyses were followed, if appropriate, by simple main effects tests. The data from rats assigned to the Footshock + sham condition were included, for comparison, in both Figures 20 a and b and seperate ANOVAs were performed using these data.

## Results

Figure 15 A illustrates the effect of footshock-stress on pain responses in the formalin test. As shown, exposure to footshock-stress 19 minutes after a formalin injection caused a strong suppression of pain responses. There was a significant main effect of Treatment, $F(1,8) = 20.7$, $p < 0.005$, and interaction between Treatment and Time, $F(17,136) = 3.16$, $p < 0.0001$. The differences between footshock and no footshock conditions were significant at all times following exposure to stress, except at 18, 24, 27, 42, and 51 minutes as revealed by simple main effects tests.

The effect of intra-VTA WIN 51708 on stress-induced analgesia is illustrated in Figure 15 B. Footshock + WIN 51708-treated rats showed a weak reversal of stress-induced analgesia throughout the course of testing, as compared to footshock + sham-treated rats. This reversal, however, was not significant; there was neither a significant main effect of Treatment (Footshock + WIN 51707 vs Footshock + sham), $F(1,15) = 2.9$, $p = 0.11$, nor a significant Treatment x Time interaction, $F(17,255) = 0.9$, $p = 0.57$. Nevertheless, simple main effects tests revealed that there were significant differences

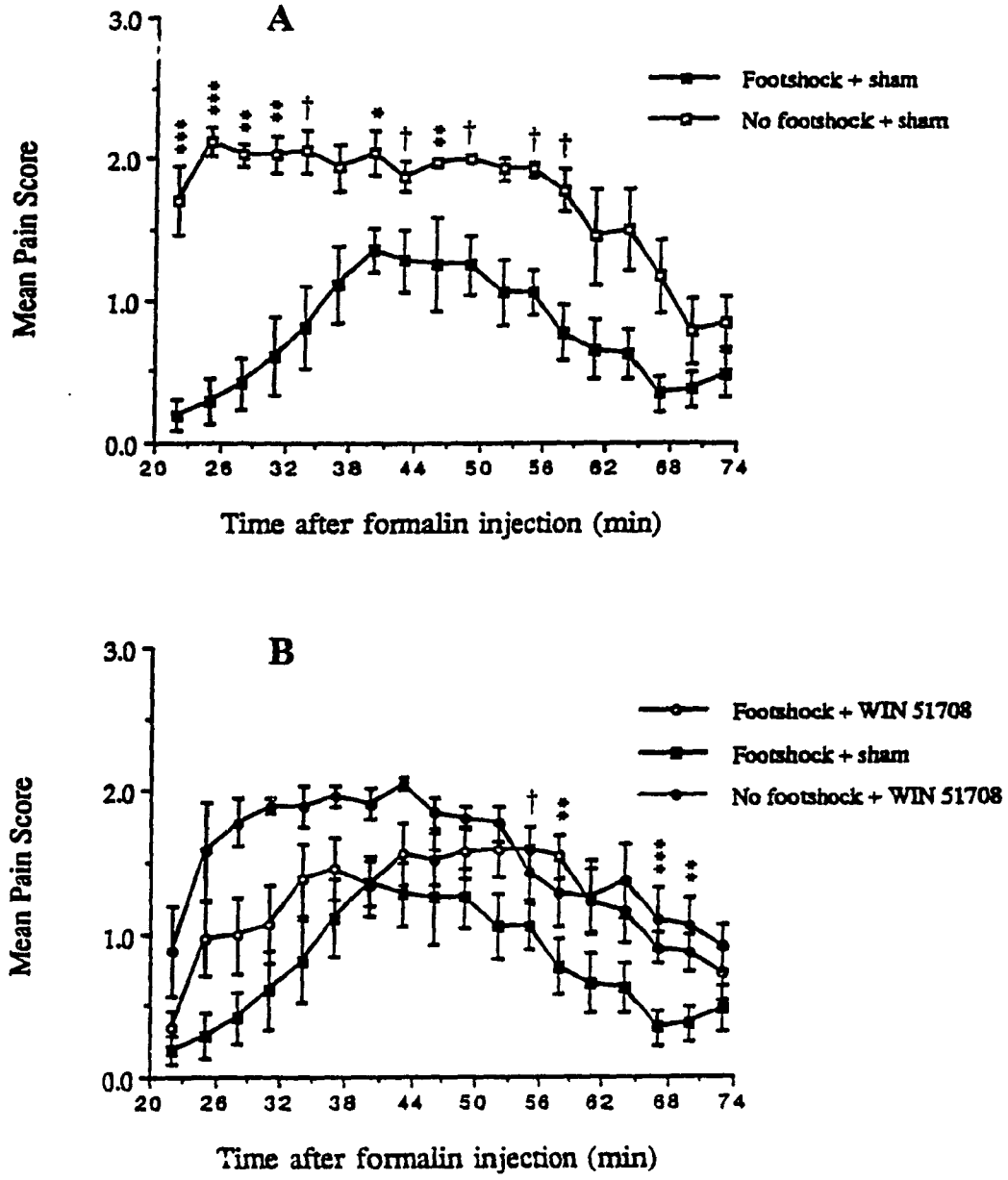


Figure 15. (A) Effect of footshock stress on mean formalin pain scores (± S.E.M). Mean pain scores differed significantly between footshock and no footshock conditions at all times following exposure to stress, except at 18, 24, 27, 42, and 51 minutes : * $p < 0.05$; † $p < 0.025$; ** $p < 0.01$; *** $p < 0.0025$. (B) Effect of intra-VTA applications of the NK-1 tachykinin receptor antagonist, WIN 51708, on stress-induced analgesia and on mean formalin pain scores (± S.E.M). There were significant differences between Footshock + WIN 51708 and No footshock + WIN 51708 conditions 36, 39, 45, and 48 minutes following exposure to stress: † $p < 0.05$; ** $p < 0.0125$; *** $p < 0.01$. Animals (n=24) were tested in a counterbalanced between-subjects design.

between WIN 51708 and sham conditions 36, 39, 45, and 48 minutes following exposure to footshock.

Figure 15 B also depicts the effect of intra-VTA WIN 51708, given in the absence of footshock, on pain responses in the formalin test. No footshock + WIN-treated rats showed an attenuation of formalin pain responses during the first few minutes of the late pain phase, as compared to No footshock + sham-treated rats. Indeed, although there was no significant Treatment (No footshock + WIN 51708 vs No footshock + sham) x Time interaction, $F(17,136) = 1.2$, $p = 0.28$, there was a significant simple main effect between these conditions 23 minutes following the formalin injection.

The location of the internal injector cannulae tips of rats tested in this experiment is illustrated in Figure 16. A total of 24 rats had their injector tips within the VTA. Three rats failed to satisfy the criterion for inclusion and, hence, their data were discarded.

## Discussion

The results indicate that blockade of (NK-1) SP receptors in the VTA caused a weak reversal of footshock stress-induced analgesia in the formalin test. This finding suggests that NK-1 receptors in the VTA mediate, in part, stress-induced analgesia in this test. The failure to observe a more pronounced reversal of stress-induced analgesia in response to intra-VTA (NK-1) SP receptor antagonism may have been due to the poor diffusion of WIN 51708 into brain tissue.

A more likely explanation for this failure is that the tachykinin receptor of interest was not blocked. Several lines of evidence suggest that NK-3, rather that NK-1, tachykinin receptors mediate the effects of neurokinins in the VTA. Although SP preferentially stimulates NK-1 receptors (Regoli *et al.*, 1988), the SP analogue, DiMe-C7, binds preferentially to NK-3 receptors (Sandberg *et al.*, 1981). In addition, studies using a highly selective NK-3 receptor ligand indicate that NK-3 binding sites are localized in the midbrain (Dam *et al.*, 1990; Stoessl & Hill, 1990) and, more precisely, on DA neurons

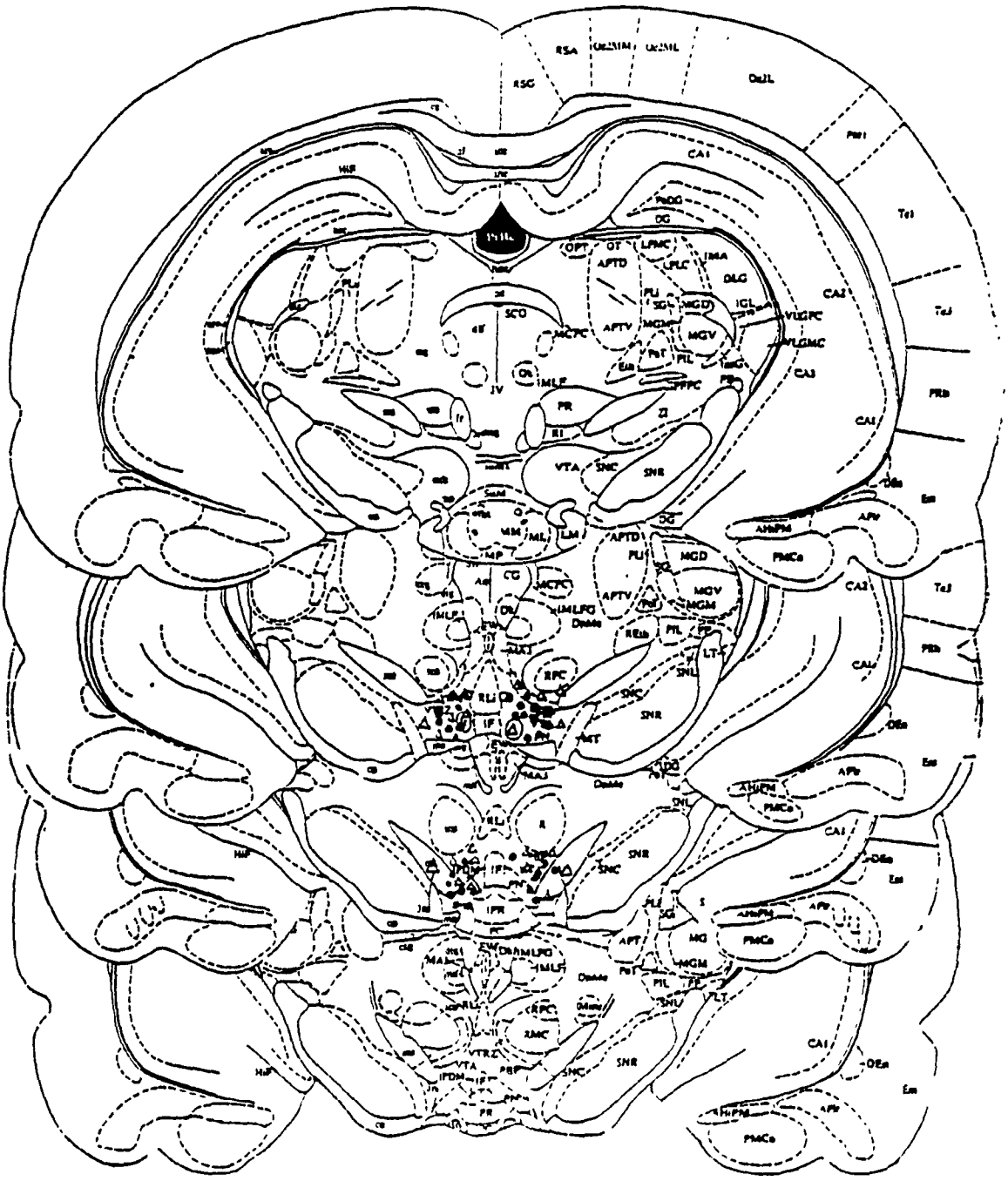

**Figure 16.** Location of the internal injector cannulae tips in the VTA of rats that received WIN 51708 in a solid crystal form (circles), or sham manipulations (triangles). Drawings are from the atlas by Paxinos and Watson (1986).

(Stoessl, 1992). Finally, DA-dependent hyperactivity can be elicited by intra-VTA infusions of a selective NK-3, but not NK-1, receptor agonist (Stoessl *et al.*, 1991) as well as by that of DiMe-C7 (e.g. Eison *et al.*, 1982a,b).

## EXPERIMENT 7

## Effects of Intra-VTA Applications of the NK-3 Tachykinin Receptor Antagonist, R-486, on Stress-Induced Analgesia in the Formalin Test

The purpose of the following experiment was to examine the role of NK-3 receptors on stress-induced analgesia in the formalin test. This was accomplished using a novel NK-3 tachykinin receptor antagonist, R-486, recently synthesized in Professor Domenico Regoli's laboratory at the University of Sherbrooke (Drapeau *et al.*, 1990; Regoli *et al.*, 1991, 1993a,b).

### Method

*Apparatus*

Apparatus were identical to those used in Experiment 6.

Surgery

Surgeries were performed exactly as described in Experiment 6.

*Drugs*

The peptide NK-3 tachykinin receptor antagonist, R-486 (generously supplied by Prof. Domenico Regoli), was used. [$Trp^7$, $\beta Ala^8$] - NKA (4-10)] (R-486) exerts good antagonist activities on the NK-3 receptor of the rat portal vein but also weak agonist properties on NK-1 and NK-2 preparations (Drapeau *et al.*, 1990; Regoli *et al.*, 1991, 1993b). The compound was either applied bilaterally to the VTA in crystalline form

packed in 28 gauge internal injector cannulae or dissolved in acid saline (pH = 6.05) and infused bilaterally in the VTA at a dose of either 0.03 or 3.0 μg/0.5 μl/side. In the latter preparation, stock concentrations (either 0.12 or 12.0 μg/2 μl) of R-486 and the vehicle, acid saline, were aliquoted in polypropylene vials and frozen at -70 ° C until used. Solutions were thawed within 30 minutes of use and were vortexed immediately prior to being drawn up into the internal injector cannulae. Complete solubility of the highest dose of R-486 (3.0 μg/0.5 μl) in acid saline was not achieved.

*Design and procedure*

The procedure used to test the rats that received bilateral intra-VTA applications of R-486 in crystalline form was identical to that employed in Experiment 6. Thus, the compound was applied to the VTA 30 minutes prior to testing. Rats were tested in a between-subjects design and were randomly assigned to one of four groups : footshock + R-486; no footshock + R-486; footshock + sham; no footshock sham. The procedure used to test rats that received microinfusions of the compound was as follows. Rats were given a formalin injection into one hind paw followed, 10 minutes later, by intra-VTA infusions of R-486 (either 0.03 or 3.0 μg/0.5 μl/side), or the vehicle. Five minutes following the intracranial infusions , rats were placed in the footshock chambers and treated thereafter exactly as described in Experiment 6. Thus, rats were tested in a between-subjects design and were randomly assigned to one of three groups : footshock + R-486 (0.03 μg/side); footshock + R-486 (3.0 μg/side); footshock + vehicle.

*Statistical analyses*

The data were analyzed by two-way ANOVAs with Treatment (2 levels; see results for details) as the between-subjects variable and Time (18 blocks of 3 minutes) as the within-subjects variable. These analyses were followed, if appropriate, by simple main effects tests. The data presented in Figure 17 from rats assigned to the Footshock + sham and No footshock + sham conditions were derived from Experiment 6 and statistical analyses were conducted using these data.

## Results

Figure 17 illustrates the effect of R-486 applied to the VTA in crystalline form on stress-induced analgesia. As shown, Footshock + R-486-treated rats failed to show a reversal of stress-induced analgesia throughout the course of testing, as compared to Footshock + sham-treated rats. There was no significant main effect of Treatment (Footshock + R-486 vs Footshock sham), $F(1,8) = 0.17$, $p = 0.69$, nor was there a significant interaction between Treatment x Time, $F(17, 136) = 0.92$, $p = 0.55$.

The effect of intra-VTA R-486 on pain responses in the formalin test is also illustrated in Figure 17. As shown, the NK-3 antagonist, given in the absence of footshock, attenuated pain responses during the latter portion of the late pain phase, as compared to the No footshock sham condition. This effect was insignificant, however, for there was neither a significant main effect of Treatment (No footshock + R-486 vs No footshock + sham), $F(1,6) = 2.9$, $p = 0.14$, nor a significant Treatment (ibidem) x Time interaction, $F(17,102) = 0.74$, $p = 0.75$.

Figure 18 A illustrates the effect of intra-VTA infusions of R-486 at a dose of 0.03 μg/0.5 μl/side on stress-induced analgesia in the formalin test. At this dose, there were no differences in formalin pain responses between R-486 and vehicle conditions throughout testing. There was no significant main effect of Treatment (R-486 at 0.03 μg/side vs vehicle), $F(1,6) = 0.01$, $p = 0.92$, nor was there a significant Treatment (ibidem) x Time interaction, $F(17, 102) = 0.79$, $p = 0.69$.

As shown in Figure 18 B, the higher dose (6.0 μg/0.05 μl/side) of R-486 attenuated the analgesic effect of footshock stress. There was a significant Treatment (R-486 at 6.0 μg/side vs vehicle) x Time interaction, $F(17, 136) = 1.97$, $p < 0.05$. Simple main effects tests indicated that the differences between this dose of R-486 and the vehicle were significant 6 and 27 minutes following exposure to footshock stress.

The location of the internal injector cannulae tips of rats tested in this experiment is illustrated in Figure 19. Of all rats that received applications of R-486 in crystalline

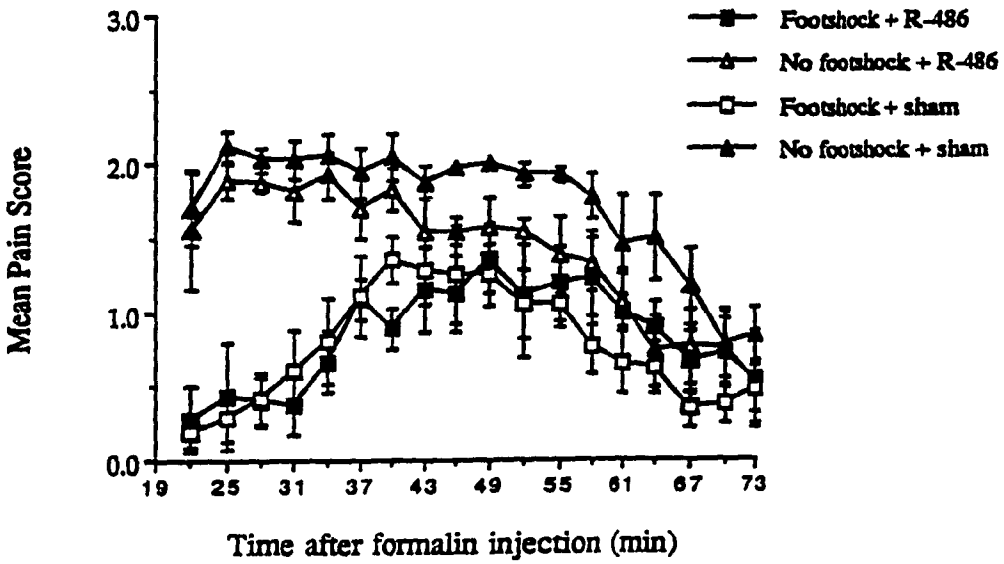


Figure 17. Effect of the NK-3 tachykinin receptor antagonist, R-486, applied in the VTA in a solid crystal form on stress-induced analgesia and on pain responses in the formalin test. Animals were tested in a counterbalanced between-subjects design.

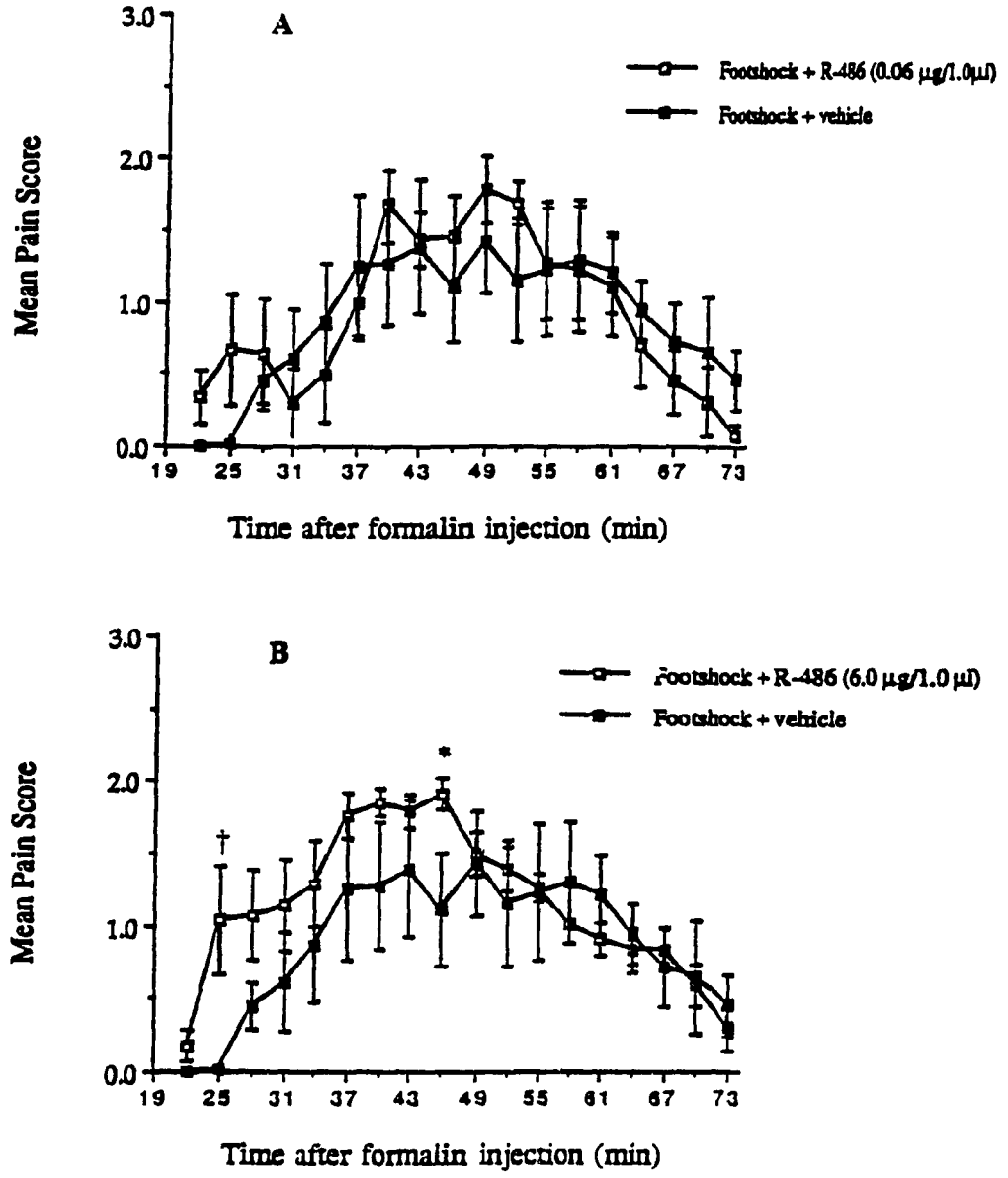


Figure 18. Effect of the NK-3 tachykinin receptor antagonist, R-486, infused into the VTA at a dose of either (A) 0.03 µg/0.5 µl/side, or (B) 3.0 µg/0.5 µl/side. This dose significantly reversed stress-induced analgesia 6 and 27 minutes following exposure to stress : † $p < 0.05$; * $p < 0.01$. Animals (n=8) were tested in a counterbalanced between-subjects design.

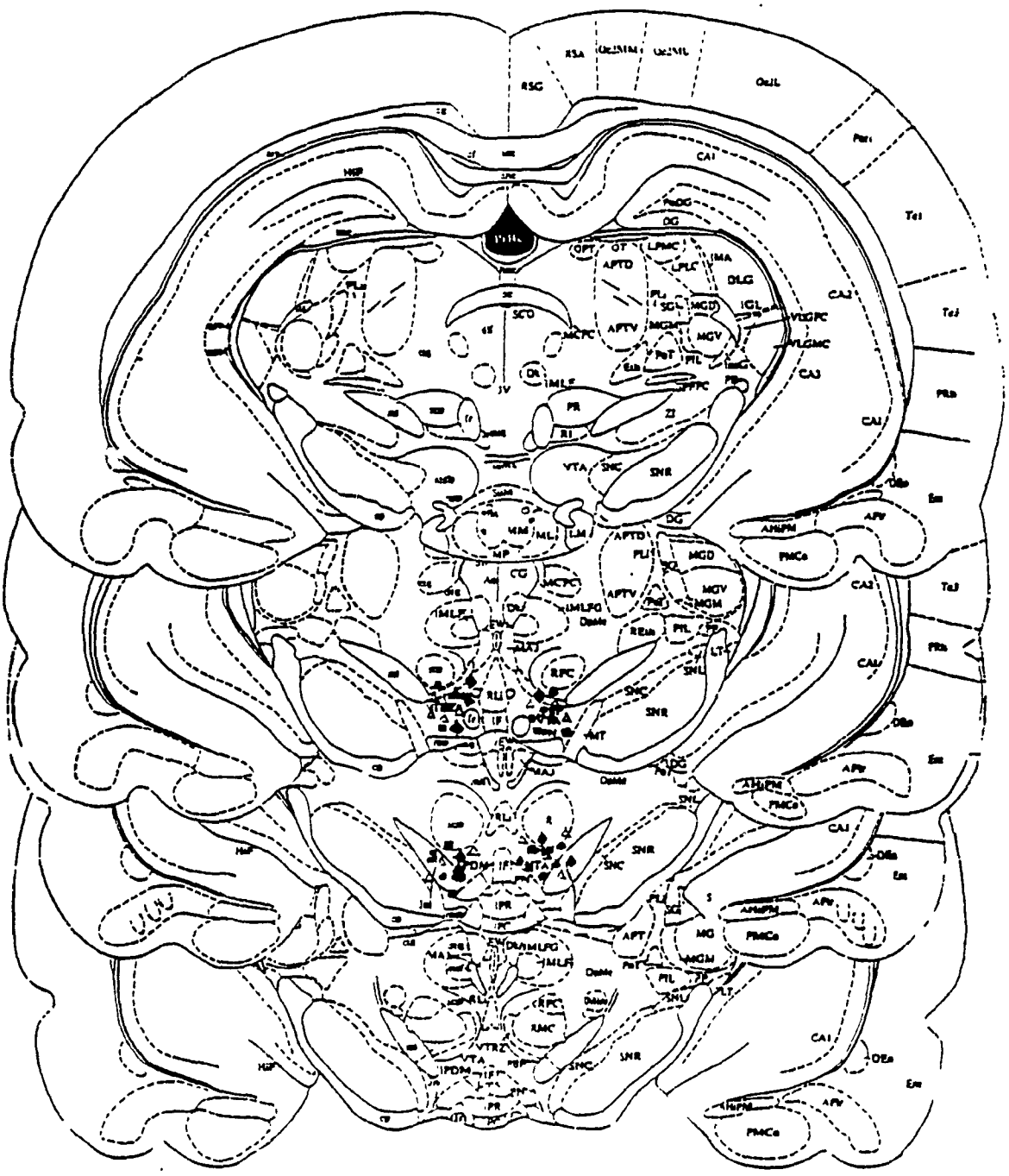

Figure 19. Location of the internal injector cannulae tips in the VTA of rats that received R-486 in a solid crystal form (triangles), microinfusions of 0.03 µg/0.5µl/side of R-486 (squares), microinfusions of 3.0 µg/0.05µl/side of R-486 (circles), or sham manipulations (diamonds). Drawings are from the atlas by Paxinos and Watson (1986).

form, 8 had their injector tips within the VTA. Of the 16 rats that received microinfusions of R-486, 14 had their injector tips within the VTA.

## Discussion

The results indicate that the NK-3 receptor antagonist, R-486, applied in a solid crystal form to the VTA, had no effect on stress-induced analgesia. Problems with the diffusion of the compound into the brain may account for the lack of effect observed under this condition. When infused in solution into the VTA, the NK-3 antagonist had no effect on stress-induced analgesia at a dose of 0.06 μg/rat, but caused a weak reversal of this response at a higher dose of 6.0 μg/rat. These findings suggest that stimulation of NK-3 receptors in the VTA mediates, in part, stress-induced analgesia in the formalin test.

The magnitude of the reversal of stress-induced analgesia by NK-3 receptor antagonism was expected to be greater given the findings that NK-3 receptors are located on DA neurons in the midbrain (Stoessl, 1992) and analgesia in the formalin test can be produced by intra-VTA infusions of the NK-3 agonist, DiMe-C7 (Experiment 1). There are several possible reasons why intra-VTA infusions of the NK-3 antagonist failed to produce a greater reversal of stress-induced analgesia. First, at the highest dose, the compound did not completely dissolve and thus it is possible that not all crystals were drawn up into the internal injector cannulae and subsequently infused into the VTA. Second, although unlikely, the dose used may not have been sufficient. Finally, it is possible that stress-induced analgesia is mediated by the joint stimulation of NK-3 and opioid receptors in the VTA, since the latter recptors play a role in the stress-induced activation of midbrain DA neurons (Kalivas & Abhold, 1987).

## EXPERIMENT 8

## Effects of the Opioid Receptor Antagonist, Naltrexone Methylbromide, on Stress-Induced Analgesia in the Formalin Test

Several lines of evidence suggest that stimulation of opioid receptors in the VTA might mediate stress-induced analgesia in the formalin test. Both peripheral (Miller *et al.*, 1984) and intra-VTA (Kalivas & Abhold, 1987) injections of opioid receptor antagonists prevent footshock stress-induced activation of DA metabolism in mesocorticolimbic neurons. The evidence that opioid receptors in the VTA play a role in the stress-induced activation of DA neurons is further supported by the findings that intra-VTA infusions of morphine and opioid analogues stimulate DA-dependent locomotor activity and increase DA metabolism in mesocorticolimbic terminal fields (Joyce & Iversen, 1979; Joyce *et al.*, 1981; Kalivas *et al.*, 1983; Kalivas & Richardson-Carlson, 1986; Kelley *et al.*, 1980). Recently, Morgan (1990) reported that intra-VTA infusions of the opioid antagonist, naloxone methylbromide, block the analgesic effects of systemic morphine in the formalin test, suggesting that morphine interacts with opioid receptors in the VTA to produce analgesia in this pain test.

The evidence that opioid receptors in the VTA mediate the stress-induced biochemical activation of midrain DA neurons and analgesia in the formalin test suggests that they might be involved in stress-induced analgesia in the formalin test. The finding that footshock stress-induced analgesia, induced by the same parameters as those used in the present thesis, is completely reversed by systemic administration of the opioid antagonist naloxone (Fanselow, 1984) supports this idea.

In the present experiment , the possibility that opioid receptors in the VTA are involved in the mediation of footshock stress-induced analgesia was examined by infusion of the opioid receptor antagonist, naltrexone methylbromide, into the VTA.

## Method

*Apparatus*

Apparatus were identical to those employed in Experiment 6.

*Surgery*

Surgeries were performed exactly as described in Experiment 6.

*Drugs*

Naltrexone methylbromide (NMB) was dissolved in saline and infused bilaterally in the VTA at a dose of 0.1 μg/0.5 μl/side. This dose of NMB was found previously to prevent the stress-induced rise in DA metabolism in mesocorticolimbic neurons (Kalivas & Abhold, 1987). In addition, intra-VTA NMB infusions were timed so that their effects in the tonic phase of the formalin test correspond to the time at which they prevent the stress-induced activation of DA neurons (Kalivas & Abhold, 1987).

*Design and procedure*

On the test day, rats were given a formalin injection into one hind paw followed, 10 minutes later, by intra-VTA infusions of NMB (0.1 μg/0.5 μl/side) or the vehicle, saline. Five minutes following the intracranial infusions, rats were placed in the footshock chambers and treated thereafter exactly as described in Experiment 6. Thus, rats were tested in a between-subjects design and were randomly assigned to one of four groups : footshock + NMB; footshock + saline; no footshock + NMB; no footshock + saline.

*Statistical Analyses*

The data were analyzed by two-way ANOVAs with Treatment (2 levels; see results for details) as the between-subjects variable and Time (18 blocks of 3 minutes) as the within-subjects variable. These analyses were followed, if appropriate, by simple main effects tests. The data from rats assigned to the No footshock + saline condition

were included, for comparison, in both Figures 27 a and b and separate ANOVAs were conducted using these data.

## Results

Figure 20 A illustrates the effect of intra-VTA NMB on stress-induced analgesia in the formalin test. As shown, NMB attenuated analgesia during the first 25-30 minutes following exposure to footshock stress. There was a significant Treatment (Footshock + NMB vs Footshock + saline) and Time interaction , $F(17,85) = 2.4$, $p < 0.005$. Tests for simple main effects revealed significant differences between NMB and saline footshock conditions at 6, 9, 12, 15, 18 and 25 minutes following exposure to footshock stress.

Figure 20 B illustrates the effect of intra-VTA NMB, given in the absence of footshock, on pain responses in the formalin test. The opioid antagonist had no effect on pain responses except during the latter part of testing. There was a significant Treatment (No footshock + NMB vs No footshock + saline) x Time interaction, $F(17, 153) = 1.8$, $p < 0.05$, and significant simple main effects between the two conditions 67, 70, and 73 minutes following the formalin injection.

The location of the injector cannulae tips is illustrated in Figure 21. As shows, 18 rats had their injector tips within the VTA. The data from 2 rats were discarded because their injector tips were outside the limits of the VTA.

## Discussion

The present results indicate that intra-VTA infusions of the opioid antagonist, NMB, block footshock stress-induced analgesia in the formalin test. The reversal of stress-induced analgesia was complete 24 to 30 minutes following intra-VTA NMB infusions. This time corresponds closely to that (i.e. 30 minutes ) following which these infusions block the stress-induced activation of midbrain DA neurons (Kalivas & Abhold,

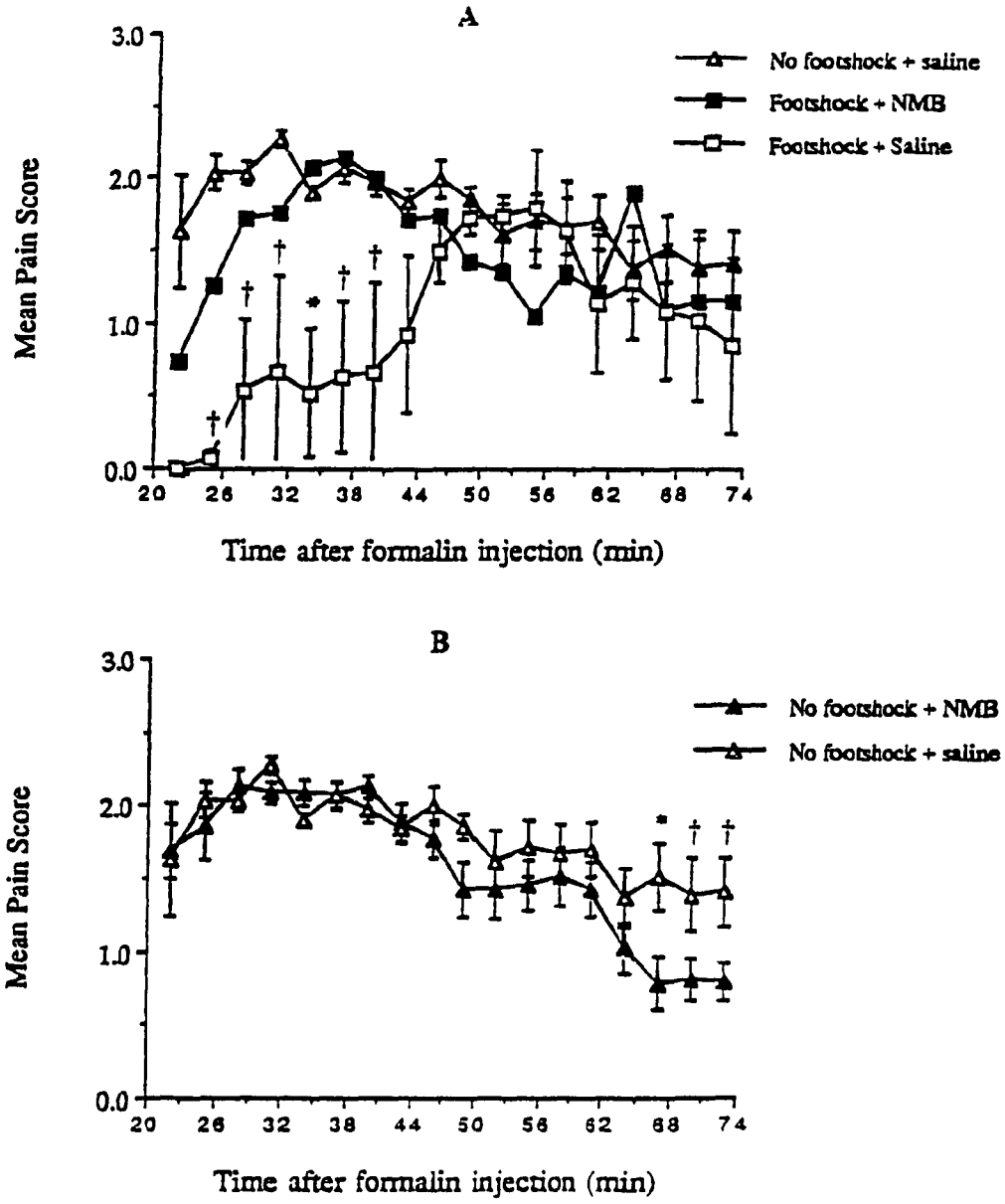


Figure 20.. Effect of the opioid antagonist, naltrexone methylbromide (NMB), infused in the VTA at a dose of 0.1 µg/0.5 µl/side on (A) stress-induced analgesia (significant differences between Footshock + NMB and Footshock + saline : † $p < 0.05$; * $p < 0.0125$) and on (B) mean formalin pain scores (± S.E.M). Significant differences between No footshock + NMB and No footshock + saline conditions : * $p < 0.01$; † $p < 0.05$. Animals (n=18) were tested in a counterbalanced between-subjects design.

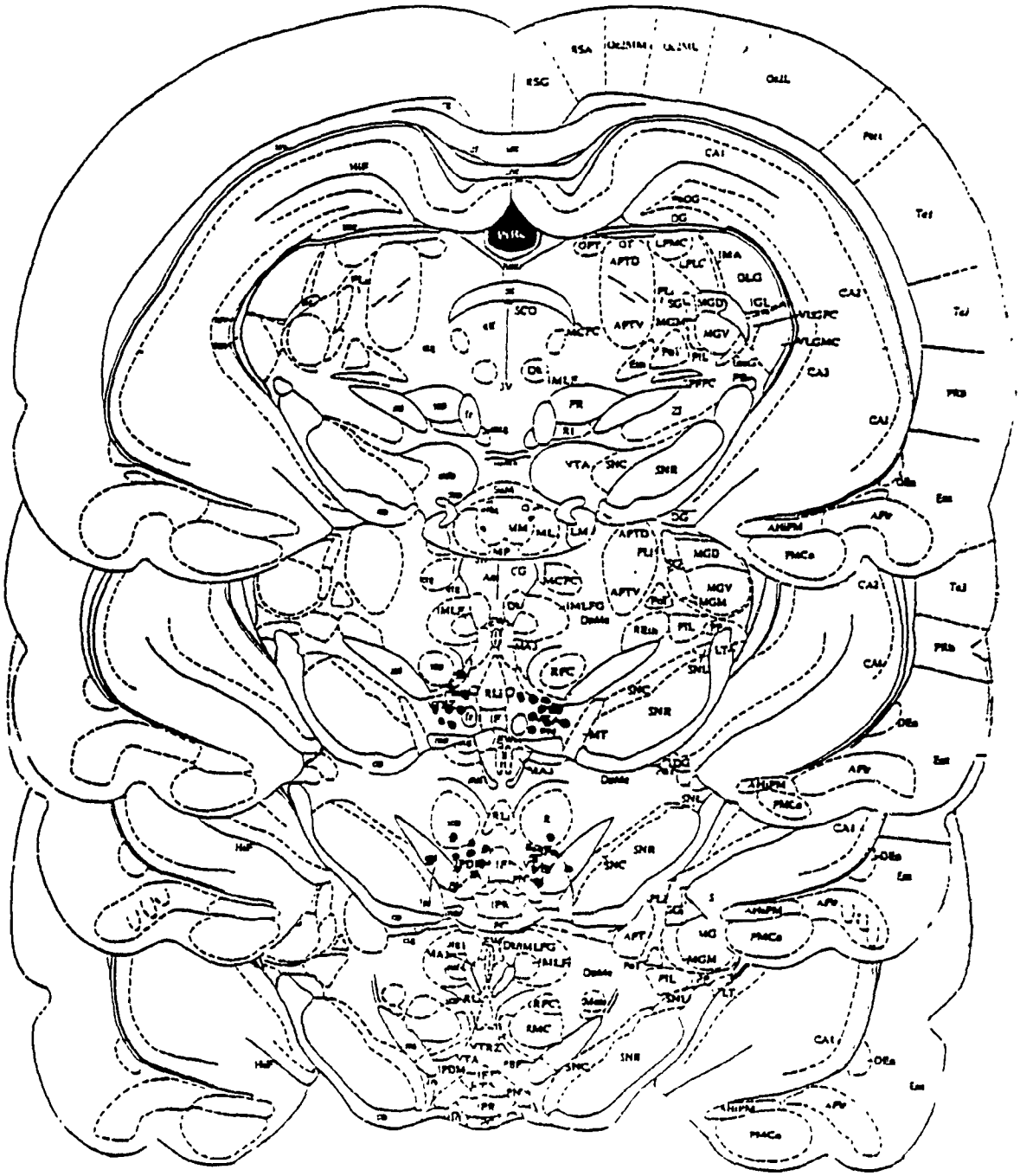

**Figure 21.** Location of the internal injector cannulae tips in the VTA of rats that received intra-VTA infusions of naltrexone methylbromide, or the vehicle. Drawings are from the atlas by Paxinos and Watson (1986).

1987). Taken together, these findings indicate that stimulation of opioid receptors in the VTA plays a critical role in mediating stress-induced analgesia in the formalin test and provide evidence that activation of midbrain DA systems is an important element for the mediation of this response. These results are consistent with those of Fanselow (1984), who showed that exposure to three 0.75 sec, 1 mA shocks delivered 20 secapart (as in the present study) caused a naloxone-reversible analgesia and extend his finding by indicating that the neuroanatomical locus underlying this opioid-mediated stress-induced response is in the VTA.

Both *mu* and *delta* receptors are present in the VTA (Moskowitz & Goodman, 1984). Because NMB is a mixed *mu* and *delta* antagonist, stimulation of either receptor subtype is responsible for mediating stress-induced analgesia in the formalin test. There is some evidence, however, to suggest that *mu* receptors may be more important for stress-induced analgesia than *delta* receptors. Latimer et al (1987) found that intra-VTA infusions of a selective *mu* agonist are more effective at stimulating DA-dependent locomotor activity and DA metabolism in mesocorticolimbic neurons than a selective *delta* agonist or a mixed agonist to these receptor subtypes.

Several lines of evidence suggest that the opioid receptors that mediate stress-induced analgesia are stimulated by enkephalin release in the VTA. There is a dense enkephalinergic innervation to the VTA (Johnson *et al.*, 1980; Khatchaturian *et al.*, 1983; Uhl *et al.*, 1978) and studies indicate that enkephalin has an excitatory effect on midbrain DA neurons. Thus, intra-VTA infusions of enkephalin analogues stimulate DA-dependent locomotor activity and increase DA metabolism in mesocorticolimbic neurons (Joyce & Iversen, 1979; Joyce *et al.*, 1981; Kalivas *et al.*, 1983; Kalivas & Richardson-Carlson, 1986; Kelley *et al.*, 1980). More recently, Kalivas and Abhold (1987) reported that enkephalin is released in the VTA in response to footshock stress and that this opioid peptide plays an important role in the stress-induced activation of DA ascending neurons.

These findings suggest that exposure to footshock stress produces analgesia by causing enkephalin release in the VTA.

In summary, the results derived from the present experiment indicate that stimulation of opioid receptors in the VTA plays a critical role in mediating stress-induced analgesia in the formalin test. Inferences from the literature suggest that exposure to footshock stress produces analgesia via the stimulation of *mu* receptors by enkephalin in the VTA.

## GENERAL DISCUSSION

In the experiments presented in this thesis it was found that activation of midbrain ascending DA neurons by application of the SP analogue, DiMe-C7, to the cell body region in the VTA decreased pain responses in the formalin test for tonic pain. Furthermore, DA release and reuptake blockade by amphetamine in the NAS, but not in the mPFC, induced similar effects. These findings provide support for the view that midbrain DA systems play a role in the suppression of tonic pain (Clarke & Franklin, 1992; Franklin, 1989; Morgan, 1990; Morgan & Franklin, 1990) and suggest that the activation of these systems by stress and noxious stimuli is a mechanism for stress-induced inhibition of tonic pain. This idea may provide a resolution to the paradox that both appetitive and aversive stimuli activate these midbrain DA systems.

It was hypothesized that SP release in the VTA and the subsequent release of DA in forebrain structures in the NAS and mPFC might underlie stress-induced analgesia in the formalin test for tonic pain. This idea was based on the evidence that stress inhibits tonic pain and causes SP-induced activation of midbrain DA systems and that analgesia can be induced in the formalin pain test by the activation of these systems. The results derived from Experiments 6 and 7 indicate that intra-VTA blockade of NK-1 and NK-3 receptors (with which SP and SP analogues interact, respectively) partially reversed stress-induced analgesia in the formalin test. These results suggest that SP release and stimulation of both NK-1 and NK-3 receptors in the VTA mediate, in part, stress-induced analgesia in the formalin test. Unfortunately, because of problems with the solubility of the compounds, these findings are preliminary and provide little information about the potency of these compounds or the relative contribution of NK-1 and NK-3 tachykinin receptors in stress-induced analgesia.

As mentioned in the Introduction, exposure to stress induces analgesia that is either opioid or non-opioid-mediated and the neurochemical nature of stress-induced analgesia is determined by several variables, the most important being stress severity (intensity x duration). In Experiment 8, it was found that intra-VTA infusions of the opioid receptor antagonist, naltrexone methylbromide, at a dose (0.2 μg/rat) found previously to prevent the stress-induced activation of midbrain DA systems, completely reversed stress-induced analgesia. Together with the findings on the NK-1 and NK-3 receptor antagonists, these results indicate that both opioids and tachykinins contribute to the stress-induced analgesia observed in the present studies.

As mentioned previously, the incomplete reversal of stress-induced analgesia by intra-VTA tachykinin receptor antagonism might be related to the antagonists used and their low degree of solubility. Alternatively, it is possible that blockade of tachykinin receptors in the VTA was not as effective in reversing stress-induced analgesia because exposure to footshock stress, using the present parameters, was ineffective in causing SP release in the VTA. As mentioned in the Introduction, exposure to footshock stress activates midbrain DA systems and it has been shown that SP release in the VTA is a critical mediator of this response (Bannon et al., 1983). Because the footshock parameters used in the present study were different from those of Bannon et al.'s study, it is not known whether SP was successfully released in the VTA. In fact, the finding that intra-VTA blockade of opioid receptors completely reversed stress-induced analgesia suggests that exposure to footshock stress, using the present parameters, caused analgesia that was mediated to a greater extent by the release of opioids than SP in the VTA. In order to have increased the chance that exposure to footshock stress caused SP release at this site and the subsequent activation of midbrain DA systems, it would have been profitable to either employ the footshock parameters in Bannon et al.'s study or to expose animals to more severe footshock stress known to cause non-opioid mediated analgesia.

It has been shown previously that SP induces naloxone-reversible analgesia

(Frederickson et al., 1978; Malick & Goldstein, 1978; Mohrland & Gebhart, 1979; Oehme et al., 1980; Stewart et al., 1976; Szreniawski et al., 1979). Since SP neither binds to opioid receptors (Onoki et al., 1977; Szreniawski et al., 1979; Terenius, 1975) nor acts like opioids on isolated tissue preparations (Frederickson et al., 1978), it has been proposed that SP produces analgesia indirectly by releasing endogeneous opioid peptides (Frederickson et al., 1978; Malick & Goldstein, 1978). Subsequent studies found that SP-induced analgesia, in a pain test similar to the formalin test, was blocked by intraventricular infusions of the antibody against met-enkephalin (Naranjo et al., 1982 a,b), and that the amount of met-enkephalin released from the periacqueductal gray correlated with the analgesic potency of SP and DiMe-C7 (Del Río et al., 1983). Several lines of evidence suggest that when SP acts in the VTA, it neither induces analgesia nor mediates stress-induced analgesia in the formalin test indirectly via opioid mecahnisms. First, SP afferents make direct synaptic contacts with DAergic cell bodies in the VTA (Tamiya *et al.*, 1990). Second, tachykinin receptors with which SP interacts are located on DA neurons in the midbrain (Stoessl, 1992). Finally, it has been shown that intra-VTA pretreatment with naltrexone methylbromide does not attenuate the capacity of intra-VTA DiMe-C7 to increase mesocortical DA metabolism, suggesting that the SP analogue mediates this response independently from opioid mechanisms.

Perhaps the most interesting finding to emerge from the present studies is that identical manipulations of midbrain DA systems produce different effects depending on the type of pain test used. Thus, activation of midbrain DA systems by intra-VTA DiMe-C7 and intra-NAS amphetamine produced analgesia in the formalin test for tonic pain, but not in the tail-flick test for phasic pain. These findings parallel those of Morgan and Franklin (1990) who showed that midbrain DA systems are involved in mediating drug-induced analgesia in the formalin, but not in the tail-flick test, and reinforce the idea that different neural systems mediate analgesia in different types of pain tests.

There is a controversy concerning the role of SP in the modulation of pain. A number of studies have indicated that the peptide induces analgesia (Del Río & Naranjo, 1983; Frederickson *et al.*, 1978; Kotani *et al.*, 1981; Malick & Goldstein, 1978; Mészaros *et al.*, 1980; Naranjo & Del Río, 1982; Naranjo *et al.*, 1981; Starr *et al.*, 1978; Stewart *et al.*, 1976; Szreniawski *et al.*, 1979; Yeomans & Proudfit, 1992). Other investigators, however, report hyperalgesia in response to SP (Cridland & Henry, 1986, 1988; Mohrland & Gebhart, 1979; Moochhala & Sawynok, 1984; Nemeroff *et al.*, 1979; Sawynok & Roberstson, 1985; Yashpal *et al.*, 1982). Several findings indicate that the nature of the effect of SP on pain sensitivity depends on the site of application as well as on such variables as housing and pH of the vehicle (Hall & Stewart, 1983), preinjection reflex-withdrawal latencies (Hall & Stewart, 1983; Naranjo et al., 1982b; Oehme et al., 1980), and peptide dose (Naranjo et al., 1982b; Oehme et al., 1980). The present findings that the SP analogue, DiMe-C7, causes analgesia in the formalin test but hyperalgesia in the tail-flick test indicate that the effect of SP on pain modulation also depends upon the type of pain test employed.

It is interesting to speculate upon the biological significance of these opposite effects of intra-VTA SP in animals exposed to stressful situations. As mentioned in the Introduction, intra-VTA SP mimics the neurochemical response to stress. In addition, informal observations made during testing in Experiments 1, 2 and 4 suggest that intra-VTA DiMe-C7 mimics the behavioral response to stress, in that rats displayed a heightened attention and reactivity to environmental stimuli such as handling. The findings that SP causes analgesia in the formalin test but hyperalgesia in the tail-flick test suggests that, under naturally stressful conditions, SP may function, concurrently, to facilitate reflexive withdrawal and active avoidance when pain is escapable and to minimize pain when it is tonic and inescapable. Thus, by causing hyperalgesia and analgesia under phasic and tonic pain stimulation, respectively, SP may serve to prevent or

reduce tissue damage and to alleviate tonic pain, thus helping the organism cope with pain and stress more effectively.

In addition to producing analgesia in the formalin test, both intra-VTA DiMe-C7 and intra-NAS amphetamine stimulated locomotor and exploratory activity, as has previously been reported (e.g. Elliott & Iversen, 1986, Vezina *et al.*, 1991). It is possible therefore that analgesia, which is itself a measure of motor activity (e.g. licking the injured paw), was an artifact of the locomotor stimulant properties of DiMe-C7 and amphetamine. Casual observation suggests that this is not an adequate explanation of the reduction in pain-associated behaviors and it has been argued previously that analgesia in this test and motor activity are dissociable, in that activity levels do not predict the degree of analgesia (Abbott, 1981; Clarke & Franklin, 1992; Morgan, 1990).

In summary, the results derived from the experiments reported in this thesis suggest that release of the neuropeptide SP in the VTA plays a facilitatory role in stress-induced analgesia in the formalin test. Because of problems with the solubility of the tachykinin receptor antagonists used in the present experiments, these findings are preliminary and provide little information concerning the extent to which SP is involved in stress-induced analgesia. Nevertheless, these findings are important and suggest that the role of midbrain SP should be re-evaluated in both opioid- and non-opioid- stress-induced analgesia, using footshock parameters in which there is evidence that SP is released in the VTA and, more importantly, using nonpeptide tachykinin antagonists that can be dissolved for intracranial microinfusions.

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