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## Neurokinin Regulation of Midbrain Raphe Neurons: A Behavioral and Anatomical Study

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NEUROKININ REGULATION OF MIDBRAIN RAPHE NEURONS:  
A BEHAVIORAL AND ANATOMICAL STUDY

by

Joseph M. Paris

A Dissertation Submitted to the Faculty of the Graduate School  
of Loyola University of Chicago in Partial Fulfillment  
of the Requirements for the Degree of  
Doctor of Philosophy

July

1988

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## VITA

The author, Joseph Michael Paris, is the son of Samuel Angelo Paris and Theresa (Allotta) Paris. Mr. Paris was born June 10, 1961, in Rochester, New York. In October, 1984, he was married to Nancy (Degnen) Paris of Glen Ellyn, Illinois.

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## CHAPTER I

### INTRODUCTION

Central nervous system (CNS) serotonin (5-hydroxytryptamine; 5-HT) neurons appear to be involved in a number of important physiological and psychological processes, including the regulation of sleep-wakefulness cycles, emotional states, and sexual and consummatory behaviors. Dysfunctions in CNS 5-HT neurotransmission are thought to contribute to the development of eating, anxiety and depressive disorders.

CNS 5-HT perikarya are organized in distinct clusters which are found only in the mesial brain stem, in association with the raphe nuclei (Taber et al., 1960). Designated B1 - B9 (Dahlstrom and Fuxe, 1964), these 5-HT containing cell groups are the source of the indoleamine innervation of the entire neuraxis. The B1 - B3 cell groups provide the primary 5-HT innervation to the spinal cord, while the B7-B9 and, to a lesser extent, the B4-B6 cell groups are the principal origin of the 5-HT innervation of the di- and telencephalon (fore-brain). Anatomically, the B7 and B8 groups are associated with the dorsal and median midbrain raphe nuclei (DR and MR, respectively). Only about 30% and 70% of the neurons in the MR and DR, respectively, contain 5-HT (Crunelli and Segal, 1985).

It is important to note that the CNS contains low 5-HT concentrations (in the ng/g range), and that well over 90% of the 5-HT in the

body is found peripherally in the enteric nervous system and blood platelets.

Early attempts to elucidate the function of central 5-HT systems employed pharmacological and lesion techniques to deplete or reduce CNS 5-HT content. Unfortunately, these methods produce dissimilar effects on central and peripheral 5-HT levels. For instance, parenteral treatment with parachlorophenylalanine (PCPA), a tryptophan hydroxylase inhibitor, produces short-term depletion of both peripheral and central 5-HT stores. Systemic administration of parachloroamphetamine (PCA), on the other hand, results in the acute release of 5-HT and norepinephrine (NE) from nerve terminals, and long-term neurotoxic effects on 5-HT processes in the brain, but not in the spinal cord or periphery. The behavioral effects of these drugs also differ. For example, in contrast to PCA, PCPA enhances an animal's response to noxious stimuli [see Lorens (1978) for a review].

Electrolytic destruction of the DR and/or MR has been used to study the functional role of the 5-HT projections to the forebrain. These lesions produce significant reductions in forebrain 5-HT levels and major behavioral changes, including increases in locomotor activity (LMA) and abnormalities in conditioned avoidance learning (Jacobs et al., 1974; Srebro and Lorens, 1975; Geyer et al., 1976). However, subsequent lesion studies employing the selective 5-HT neurotoxin, 5,7-dihydroxytryptamine (5,7-DHT; Baumgarten et al., 1972, 1973), failed to reproduce the behavioral effects obtained following electrolytic raphe lesions (Geyer et al., 1976; Hole et al., 1976; Lorens et al., 1976;

Lorens, 1978). Thus, the depletion approach has not been successful in ascertaining the role of 5-HT in behavior.

The discovery of multiple 5-HT recognition sites (5-HT<sub>1,2,3</sub> and subtypes) has led to the development of a number of novel pharmacological tools for studying 5-HT function (Peroutka and Snyder, 1979; Goodwin and Green, 1985; Tricklebank, 1985; Peroutka, 1988). However, parenteral administration of 5-HT agonists and antagonists will affect peripheral as well as central 5-HT receptors. Thus, intracerebral drug administration appears to provide the best available means to study central 5-HT processes.

Electrophysiologic investigations suggest that raphe 5-HT neurons are modulated by local  $\gamma$ -aminobutyric acid (GABA) neurons (Gallagher and Aghajanian, 1976; Mugnaini and Oertel, 1984). Przewlocka et al. (1979) were the first to report that intra-DR injections of the GABA<sub>A</sub> agonist, muscimol, increase LMA and induce feeding. Subsequently, Sainati and Lorens (1982) found that muscimol infusions into the DR and MR dose-dependently increase LMA, and that the MR was approximately four times more sensitive than the DR to the hyperkinetic effects of muscimol. The effects of muscimol also were shown to be potentiated by benzodiazepine pretreatment (Sainati and Lorens, 1983).

Little work has been done to study the regulation of raphe cells by extrinsic neurons, such as those which release substance P (SP). The midbrain raphe has been shown to contain SP-like immunoreactive fibers, but not SP-like perikarya (Ljungdahl et al., 1979; Neckers et al., 1979). Paris and Lorens (1987 a,b) demonstrated that infusions into the MR of the metabolically stable SP analog, DiMe-C7 (Sandberg et

al., 1981), produce dose-dependent increases in LMA which can be blocked by prior treatment with PCPA and by intra-MR 5,7-DHT injections. These observations suggest that the LMA effects of intra-*raphe* DiMe-C7 administration depends on the integrity of 5-HT neurons. In contrast, the effects of intra-MR muscimol on LMA are not mediated by 5-HT neurons (Paris and Lorens, 1987 a,b; Wirtshafter et al., 1987).

Infusions of DiMe-C7 into the ventral tegmental area (VTA), just rostral to the MR, have been reported to produce locomotor hyperactivity (Eison et al., 1982 a,b). These observations suggest that the behavioral effects observed following intra-MR infusions of DiMe-C7 might be due to diffusion of the peptide to the adjacent VTA. However, it has been demonstrated that lesions of the MR produced by the excitatory amino acid neurotoxin, ibotenic acid, which destroys cell bodies but spares fibers of passage (Coyle and Schwarcz, 1983), completely prevent the effects of intra-MR DiMe-C7 and muscimol on LMA (Paris and Lorens, 1987 a). In addition, pretreatment with the dopamine (DA) antagonist, haloperidol (200  $\mu$ g/ml, i.p.), has been shown to block the hyperactivity produced by intra-MR infusions of DiMe-C7. In contrast, haloperidol does not affect the LMA effects of intra-*raphe* muscimol (Wirtshafter et al., 1987; our unpublished data).

These observations suggest that two distinct pathways involving the MR modulate behavioral arousal: one is a GABA---non-5-HT---non-DA circuit, and the second is a neurokinin---5-HT---DA circuit. This view is supported by observations that, in contrast to DiMe-C7, muscimol infusions into the MR produce dose-dependent increases in food intake and

in plasma adrenocorticotrophic hormone (ACTH) and corticosterone levels (Klitenick and Wirtshafter, 1986; Lee, J. et al., 1987).

SP belongs to a family of bioactive peptides called tachykinins. The tachykinins share the common C-terminal amino acid sequence, Phe-X-Gly-Leu-Met-NH<sub>2</sub> (Erspamer, 1981; Harmar, 1984). Three of these peptides and their messenger RNA (mRNA) precursors have been localized in the periphery and CNS of mammals: SP, neurokinin A (NKA), and neurokinin B (NKB). Non-mammalian tachykinins include eledoisin, physalamin, and kassinin.

The neurokinins possess three distinct binding sites in the CNS and periphery (Quirion, 1985). These recognition sites have been designated NK-1, NK-2, and NK-3, and correspond to the preferred binding sites for SP, NKA, and NKB, respectively (Lee, C.M. et al., 1986; Henry, 1987).

Several investigators have studied the distribution of neurokinin recognition sites in the CNS. Moderate numbers of NK-1 and NK-3 sites (measured as [<sup>125</sup>I]-Bolton-Hunter SP and eledoisin binding) have been observed in the MR and DR (Beaujouan et al., 1986; Saffroy et al., 1988). NKA-binding also has been detected in the midbrain raphe, as well as in other brain areas (Quirion and Dam, 1985; Buck et al., 1986). The existence of NK-2 recognition sites in the CNS, however, has been debated (Saffroy et al., 1988). Nevertheless, SP-like immunoreactive (SPLI) fibers, as well as NKA-immunoreactivity (Cuello and Kanazawa, 1978; Ljungdahl et al., 1978 a; Takano et al., 1987), have been discerned in the midbrain raphe.

Our preliminary studies indicated that much higher doses of SP than DiMe-C7 had to be injected into the MR in order to elicit hyperactivity. DiMe-C7, furthermore, has been recently shown to have a greater affinity for the NK-3 than for the NK-1 binding site (Regoli et al., 1987; Weinrich et al., 1987). Thus, the first major goal of the dissertation project was to determine which neurokinin(s) and receptor(s) mediate(s) the LMA effects observed following intra-MR infusion. To achieve this aim, we compared the behavioral effects of the distinct tachykinins and their analogues following their injection into the MR.

Optimally, in order to reach any conclusions concerning receptor selectivity, one should use a specific competitive antagonist to block the action of a particular group of agonists. For the tachykinins, however, this pharmacological approach has several limitations. Although a number of neurokinin antagonists have been developed (Folkers et al., 1981; Regoli et al., 1984 b), they have been shown to be neurotoxic, to have local anesthetic properties, and to have non-specific actions following their central administration in the rat (Hokfelt et al., 1981; Freedman et al., 1986; Yusof and Coote, 1987). Tachykinin antagonists have been used in behavioral studies (Elliott and Iversen, 1987). However, these authors found that the neurokinin antagonists produced extensive histological damage following their central administration into the rat (Dr. Peter J. Elliott, personal communication, 1986). On the basis of these data, neurokinin antagonists were not employed.

Two series of experiments were performed. The first series consisted of ten experiments which analyzed the behavioral effects of

tachykinin administration into the MR. The second series of experiments studied the anatomical origin of the neurokinin innervation of the MR.

The first behavioral experiments examined the dose-dependent effects of DiMe-C7 on LMA. The effects of the non-mammalian and mammalian tachykinins, as well as the selective NKB selective agonist, senktide (Laufer et al., 1986 b; Wormser et al., 1986), then were compared to the effects of vehicle and DiMe-C7. Subsequently, lower doses of NKA, senktide and NKB were studied.

The objective of the next experiment was to determine whether the hyperkinetic effects of the neurokinins are mediated by raphe 5-HT neurons and their projections to the forebrain. This was examined by producing 5,7-DHT lesions in the MR and in the 5-HT fibers which ascend in the medial forebrain bundle.

To further characterize the behavioral effects of intra-MR neurokinin infusions, rats were observed in an open field arena. In the first experiment, we injected the neurokinins into the MR, then placed the animals in a novel environment. In the second experiment, we examined the effects of the neurokinins on the animals' behavior in a familiar environment. The purpose of these studies was 1) to analyze the types of behaviors elicited by intra-MR neurokinin injections, and 2) to determine whether the behavioral patterns evoked varied as a function of the novelty of the test environment.

Although SP-like and other neurokinin-like afferents to the MR have been postulated to originate in the lateral habenular nucleus (Neckers et al., 1979), this region does not contain neurokinin-like

cell bodies (Ljungdahl et al., 1978 a). Thus, the second major goal of the dissertation project was to determine the origin of the neurokinin innervation of the MR. This was investigated by injecting the retrogradely transported fluorescent dye, Fluoro-Gold, into the MR and mapping the labeled cells. This was necessary because the afferents to the MR have not been thoroughly documented. Next, using immunocytochemical techniques, SP- and neurokinin-like fibers were localized in the raphe. Finally, by combining immunocytochemistry and retrograde tracing, an attempt was made to localize the neurokinin-containing perikarya which send axons to the MR.

Thus, the overall goals of the dissertation project were to study the regulation of median raphe 5-HT neurons by a particular group of peptides, the neurokinins, and to determine the origin of the neurokinin projections to the MR.

## CHAPTER II

### REVIEW OF THE RELEVANT LITERATURE

#### A. Midbrain Raphe Nuclei

The groups of neurons which occupy the midsagittal plane of the brain stem are referred to as the raphe nuclei. Although recognized since the earliest studies of neuroanatomy, the seminal reports concerning the nomenclature, topography, afferent and efferent connections were first published in 1960 (Brodal et al., 1960 a,b; Taber et al., 1960). Interest in the raphe nuclei was piqued after Dahlstrom and Fuxe (1964), utilizing the Falck-Hillarp formaldehyde-induced fluorescence technique, identified nine distinct clusters of 5-HT containing neurons in the brain stem raphe nuclei of the rat. These 5-HT cell groups were designated B1 - B9, according to their caudorostral position (Dahlstrom and Fuxe, 1964). The B7 and B8 5-HT cell groups overlap the midbrain dorsal and median raphe nuclei (DR and MR), respectively. These observations subsequently have been substantiated and extended using immunocytochemical techniques (see Steinbusch and Nieuwenhuys, 1983, for a review).

Perikarya also have been localized within the midbrain raphe which contain dopamine (DA; Ochi and Shimizu, 1978), GABA (Mugnaini and Oertel, 1985), cholecystokinin (Vanderhaeghen et al., 1980), met-enkephalin (Uhl et al., 1979; Murakami et al., 1987), and corticotropin-

releasing factor (CRF; Sakanaka et al., 1987). SP-containing cell bodies have been observed in the DR, but not in the MR (Ljungdahl et al., 1978; Chan-Palay et al., 1978).

#### Co-localization of Transmitters within the Midbrain Raphe

One of the most exciting but least understood aspects of neurotransmission is the coexistence of more than one neurotransmitter in the same neuron. Several questions have been raised concerning the possible co-release, pre- versus post-synaptic effects, and neurohumoral roles of the co-localized substances. In most instances, little is known beyond the anatomical information. The potential coexistence of 5-HT with other neurotransmitters has been investigated by several laboratories. Cell bodies which contain both 5-HT and GABA have been observed in various raphe nuclei, with the greatest number being found in the DR (Belin et al., 1983; Magoul et al., 1986; Harandi et al., 1987; Millhorn et al., 1987). However, these results should be interpreted cautiously. Some have relied on the combined use of immunocytochemistry with the uptake of radiolabeled 5-HT and GABA (Magoul et al., 1986; Harandi et al., 1987). False-positive staining can occur due to non-specific cellular accumulation, particularly by glial cells (Kelly and Dick, 1976). The fixation procedures used for combined autoradiography and immunocytochemistry represent a compromise between the optimal techniques for one of the methods alone, and may actually interfere with the visualization of the reaction products (Harandi et al., 1987). Similarly, the visualization of one-substance by the peroxidase-anti-peroxidase (PAP) method can interfere with the fluorescent label used

to view the other (Belin et al., 1983). The coexistence of GABA and 5-HT in the midbrain raphe is an interesting possibility, but requires additional confirmatory evidence.

Serotonin and leu-enkephalin also have been reported to coexist in the cat DR and nucleus raphe magnus (Glazier et al., 1981). The colocalization of serotonin with N-acetyl-aspartyl-glutamate, an acidic dipeptide proposed as a neurotransmitter in some putative glutaminergic pathways (Koller and Coyle, 1984), has been found in the brain stem raphe, including the rat MR and DR (Forloni et al., 1987).

#### Co-localization with Substance P

The coexistence of SP and 5-HT in the caudal brain stem raphe has been demonstrated in several species (Chan-Palay et al., 1978; Hokfelt et al., 1978; Pelletier et al., 1981). In addition, thyrotropin-releasing hormone (TRH) has been found to coexist with both SP and 5-HT in the same neuron (Johansson et al., 1981). The 5-HT/SP containing neurons send axons exclusively to the spinal cord and a great deal of work has focussed on their interactions in this region (see Holstege and Kuypers, 1987; Thor and Helke, 1987). Only one report has suggested the possibility of SP/5-HT co-localization within the DR (Magoul et al., 1986). This study relied on the use of radiolabeled 5-HT uptake, combined with SP immunocytochemistry, and has not been confirmed by other investigators.

Efferent Projections of the Midbrain Raphe

The 5-HT and non-5-HT projections of the midbrain raphe have been studied extensively. Early reports indicated that the MR and DR are the source of the 5-HT fibers which ascend in the medial forebrain bundle and innervate the forebrain (Heller and Moore, 1965; Anden et al., 1966). It also has been shown that neurons of the midbrain raphe project to almost all areas of the brain (Conrad et al., 1974; Bobillier et al., 1975; Parent et al., 1981). Although the axonal projections of the raphe nuclei overlap and are highly collateralized, they are topographically organized. For instance, the hippocampus and neostriatum receive 5-HT projections which arise predominantly in the MR and DR, respectively (Jacobs et al., 1974; Lorens and Guldberg, 1974; Geyer et al., 1976 a,b; Azmitia and Segal, 1978; Kohler and Steinbusch, 1982). There also is a great deal of evidence concerning the divergence and collateralization of raphe projections (de Olmos and Heimer, 1980; van der Kooy and Hattori, 1980; Fallon and Loughlin, 1982). Recently, Imai and colleagues (1986) have suggested that the projections of the midbrain raphe are topographically organized. These authors reported that cells positioned rostrally in the MR and DR project to the basal ganglia, while those situated caudally send axons to limbic structures. Projections to the amygdala appear to arise from a distinct group of cells in the DR (Imai et al., 1986). Thus, neurons within the midbrain raphe are in a position to modulate neuronal systems throughout the di- and telencephalon. However, the extent to which these forebrain systems reciprocally communicate with raphe neurons has not been adequately examined.

### Afferent Connections of the Midbrain Raphe

A number of transmitter-specific fibers and terminals have been localized within the midbrain raphe. These include DA, norepinephrine, GABA, cholecystokinin, neurotensin, and enkephalin (see review by Steinbusch and Nieuwenhuys, 1983). The origin of the afferent projections to the raphe in the cat were studied originally using a retrograde degeneration technique (Brodal et al., 1960 b). Since then, the retrograde transport of horseradish-peroxidase has been employed to study MR and DR afferents in the rat and rabbit, but only in comparison to those of the pontine reticular formation (Pasquier et al., 1976; Aghajanian and Wang, 1977; Hayakawa and Zyo, 1986). The origin of the afferents to the rat MR (summarized below) is based on a review of studies which focussed primarily on the efferents from various fore-brain regions. In most of these studies, the efferent projections to the MR are diagrammatically plotted, but rarely commented upon. The anterograde transport of tritiated amino acids has been the most frequent technique used. Recent studies have employed the kidney bean lectin, Phaseolus vulgaris leucoagglutinin (Gerfen and Sawchenko, 1984).

Afferents to the MR have been reported to originate in: the frontal cortex, septal region and diagonal band of Broca (Aghajanian and Wang, 1977; Swanson and Cowan, 1979; Swanson, 1982; Swanson et al., 1987; Tomimoto et al., 1987); the medial and lateral preoptic areas (Swanson et al., 1984, 1987); the lateral border of the internal capsule (Haber et al., 1985); the lateral hypothalamus (Saper et al., 1979; Swanson et al., 1984); the perifornical nucleus (Pasquier et al.,

1976); the subfornical organ (Lind, 1986); the laterodorsal tegmental nucleus (Sato and Fibiger, 1986); and, the interpeduncular nucleus (Hamill and Jacobowitz, 1984; Groenewegen et al., 1986; Shibata et al., 1986). A major fiber system from the lateral habenular nucleus to the MR has been demonstrated as well (Pasquier et al., 1976; Aghajanian and Wang, 1977). Projections from the B9 5-HT cell group to the brain stem also have been reported (Aghajanian and Wang, 1977). A thorough examination of the afferents to the MR using a retrograde tracing technique is important in order to substantiate the results reviewed above.

#### Behavioral Effects of Midbrain Raphe Lesions

Some of the earliest experiments investigating the role of fore-brain 5-HT in behavior focussed on the 5-HT depleting effects of electrolytic destruction of the midbrain raphe nuclei. Electrolytic lesions of the MR, which produce a greater than 80% reduction in hippocampal 5-HT levels, also produce significant increases in LMA (Jacobs et al., 1974). These authors suggested that the reduction in hippocampal 5-HT concentrations could account for the increase in activity. These effects were subsequently corroborated by others (Srebro and Lorens, 1975; Geyer et al., 1976). Combined MR and DR lesions also were found to facilitate avoidance acquisition in a shuttle box and a Y-maze (Lorens et al., 1971; Steranka and Barrett, 1974). More recently, electrolytic raphe lesions have been found to impair the acquisition and performance of an 8-arm radial maze task, and the reversal of a learned position habit, but not the acquisition or reversal of a simultaneous brightness discrimination task (Wirtshafter and Asin, 1983, 1986).

Subsequent lesion studies found that electrolytic destruction of the DR and MR enhanced the hyperkinetic effects of d-amphetamine (Lucki and Harvey, 1979). Studies by Asin and Fibiger (1983) indicate that lesions of the MR with ibotenic acid, a neurotoxic excitatory amino acid which destroys cell bodies in a region but spares fibers of passage (Coyle and Schwarcz, 1983), also produce hyperactivity and potentiate the locomotor stimulant effect of d-amphetamine.

These studies suggest that the midbrain raphe nuclei play an important role in several behavioral processes, but say little about the role of 5-HT neurons. Since mechanical and excitotoxic lesions of the midbrain raphe simultaneously produce reductions in forebrain 5-HT concentrations, there was a consensus that the behavioral effects were due to the degeneration of ascending 5-HT fibers. However, these types of lesions also destroy non-5-HT elements within the raphe. With the discovery and use of the serotonergic neurotoxins, 5,6-dihydroxytryptamine and 5,7-DHT (Baumgarten et al., 1972, 1973), a new method to selectively produce deficits in forebrain 5-HT was available. The potential of these neurotoxins was great since discrepancies had been noted with various other methods of depleting forebrain 5-HT (Lorens, 1978). It was found that 5,7-DHT lesions of the MR, DR or ascending 5-HT fibers, which produced significant reductions in forebrain 5-HT concentrations, did not affect activity levels or avoidance conditioning (Hole et al., 1976; Lorens et al., 1976; Asin and Fibiger, 1983). Wirtshafter and colleagues (1986) subsequently demonstrated that the LMA effects following raphe damage can be disassociated from forebrain 5-HT depletion, and suggest that a descending pathway may be involved

in the generation of MR mediated changes in LMA. It had become apparent that other techniques besides lesions were necessary to study the function of midbrain raphe 5-HT neurons.

#### Pharmacological Manipulations of Midbrain Raphe Neurons

A method developed to specifically and acutely affect the activity of raphe neurons was the microinjection of compounds directly into brain areas containing 5-HT perikarya. Several investigators have used this approach to confirm that GABA serves as an inhibitory transmitter within the raphe (Gallager and Aghajanian, 1976; Gallager, 1978; Forchetti and Meek, 1981), and that benzodiazepine/GABA interactions are important to drug induced changes in raphe function (Gallager, 1978). Przewlocka and associates (1979) demonstrated that injection of the GABA<sub>A</sub> agonist, muscimol, into the DR, produces increases in LMA and food intake. At the same time, Arnt and Scheel-Kruger (1979) demonstrated a similar effect after muscimol injections into the "caudal-VTA" (which most likely affected neurons within the rostral portion of the MR). Subsequently, Sainati and Lorens (1982) found that muscimol produced dose-dependent increases in LMA when injected into the MR and DR, and that the MR was approximately four times more sensitive. The effect of intra-MR muscimol injections also was shown to be potentiated by intra-raphe administration of benzodiazepines (Sainati and Lorens, 1983). Although originally thought to be mediated by 5-HT neurons (Sainati and Lorens, 1982), it has subsequently been demonstrated that the hyperkinetic effects of intra-MR muscimol infusions are not media-

ted by 5-HT neurons (Paris and Lorens, 1987 a,b; Wirtshafter et al., 1987 a).

It has been suggested that the projection from the lateral habenular nucleus to the MR utilizes an excitatory amino acid neurotransmitter (Kalen et al., 1985, 1986). Injection of the glutamate analogue, kainic acid, into the MR has been reported to suppress LMA (Wirtshafter and McWilliams, 1987). However, these results are somewhat equivocal since injections of N-methyl-D-aspartate (NMDA) into the MR produce explosive short bursts of hyperactivity which can be blocked by the NMDA antagonist, DL-2-amino-5-phosphonovaleric acid (APV; unpublished observations). Further studies are necessary to clarify this phenomenon. More recently, intra-MR injections of the herbicide, paraquat [an analogue of the MPTP (N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) metabolite, MPP<sup>+</sup> (N-methyl-4-phenyl-pyridinium)], have been reported to produce increases in LMA, circling, and wet-dog shakes (DeGori et al., 1988). The mechanism of these effects is not understood.

Thus, the local application of substances has demonstrated that behavioral effects can be elicited by acutely affecting the activity of raphe neurons.

#### Raphe 5-HT Neurons and Ingestive Behaviors

The role of 5-HT in food intake and eating disorders has been extensively reviewed (Sugrue, 1987; Morley and Blundell, 1988), and stems from early observations of the anorectic effect of the 5-HT releasing drug, fenfluramine (as reviewed in Rowland and Carlton,

1986). The role of raphe neurons in the regulation of food intake initially was investigated pharmacologically by Przewlocka and colleagues (1979), who reported that injections of muscimol into the DR produced increases in food intake in non-food deprived rats. Arnt and Scheel-Kruger (1979) found that muscimol injections into the "caudal VTA" (rostral MR) produce similar effects. It was reported subsequently that the hyperphagia induced by muscimol could be inhibited by (+)-fenfluramine pretreatment (Borsini et al., 1983). The involvement of 5-HT in muscimol induced hyperphagia also was suggested by the observation that 5-HT or norfenfluramine (a bioactive metabolite of fenfluramine) infusions into the nucleus accumbens could inhibit muscimol, but not starvation-induced eating in rats (Bendotti et al., 1986 b). These authors went on to suggest that muscimol's effects were modulated in part by DA neurons since the DA antagonist, fluphenazine, could attenuate intra-DR muscimol-induced eating when the antagonist was injected into either the caudate-putamen or the nucleus accumbens (Bendotti et al., 1986 a). On the other hand, it has been shown that the MR is more sensitive than the DR to the hyperphagic effects of muscimol, and that 5,7-DHT lesions of the MR or DR do not prevent the feeding induced by muscimol infusions into the raphe (Borsini et al., 1983; Klitenick and Wirtshafter, 1986, 1987). These results suggest that, like the effects of muscimol on LMA, muscimol-induced hyperphagia is mediated by non-5-HT elements within the midbrain raphe.

The results observed with muscimol are interesting in light of the fact that there is a considerable body of evidence that the 5-HT<sub>1A</sub> agonist, 8-hydroxy-2-(di-N-propylamino)-tetralin (8-OHDPAT; Arvidsson

et al., 1981; Hjorth et al., 1982) elicits feeding following its administration either peripherally or directly into the raphe (Dourish et al., 1985 a,b,c, 1986 b; Bendotti and Samanin, 1986; Hutson et al., 1986). It is postulated that these effects occur through a decrease in the firing rate of 5-HT neurons via activation of 5-HT<sub>1A</sub> somatodendritic autoreceptors (de Montigny et al., 1984; Dourish et al., 1985 a, 1986 a; Verge et al., 1985; Weissmann-Nanapoulos et al., 1985). However, controversy exists as to whether the 8-OHDPAT-induced hyperphagia is due to non-specific gnawing or to stimulation of appetite which may counter a tonic 5-HT inhibitory influence on feeding (Fletcher, 1987; Dourish et al., 1988).

#### Raphe 5-HT Neurons and Anxiety

The role of 5-HT in anxiety, and in the anxiolytic effects of the benzodiazepines, has been extensively reviewed and contested in recent years (Gardner, 1986; Johnston and File, 1986; Soubrie, 1986; Thiebot, 1986). The midbrain raphe has been analyzed in terms of the effects of benzodiazepine/GABA/5-HT interactions (Thiebot et al., 1980, 1982, 1984; Sainati and Lorens, 1983; Nishikawa and Scatton, 1983; Green and Hodges, 1986; Mendelson et al., 1987). Intra-DR injections of the benzodiazepine inverse agonist, methyl- $\beta$ -carboline-carboxylate ( $\beta$ -CCM), reduce social interaction in pairs of rats, suggesting an anxiogenic-type of effect (File and Hyde, 1978; File et al., 1982; Guy and Gardner, 1985; Jones et al., 1986).

Clinically, interest in the role of 5-HT neurons in the control of anxiety has been heightened by the recent Food and Drug Administra-

tion approval (1987) of buspirone (BuSpar<sup>R</sup>), a non-benzodiazepine azaspirodecanedione, approved for use in the treatment of anxiety (Tayler et al., 1985). Buspirone binds with a high affinity to 5-HT<sub>1A</sub> receptors (Peroutka, 1985), and decreases the activity of DR 5-HT neurons (Vander Maelen et al., 1986). Since buspirone also has been reported to have DA binding properties (Riblet et al., 1982), an analogue of buspirone, gepirone, which is devoid of DA binding properties, but has a high affinity for 5-HT<sub>1A</sub> receptors (Yocca et al., 1986; see Blier and de Montigny, 1987), has been studied. Electrophysiologically, gepirone also decreases the firing rate of 5-HT neurons (Blier and de Montigny, 1987), and is efficacious in the treatment of anxiety (Cott et al., 1986; Csanalosi et al., 1987). Furthermore, 8-OHDPAT has been reported to have anxiolytic properties in rats (Carli and Samanin, 1988). Thus, the data indicate that 5-HT circuits play a role in anxiety, and in the mechanism of action 5-HT<sub>1A</sub> anxiolytics, as well as the benzodiazepines.

### B. Neurokinins

Since its discovery by von Euler and Gaddum (1931), the bioactive peptide, substance P (SP), has been the subject of a considerable amount of research (see reviews by Maggio, 1988; Nicoll et al., 1980; Pernow, 1983). Recent work, however, has focused upon the family of peptides related to SP, called the "tachykinins." These were so-named because they have a characteristic fast onset of action on gut tissue, in comparison to the much slower acting bradykinins.

### Nomenclature

As shown in Table I, the tachykinins share a common carboxy-terminal sequence  $\text{-Phe-X-Gly-Leu-Met-NH}_2$ , where X can be an aromatic or branched aliphatic amino acid (Erspamer, 1981, Harmar, 1984). Three of the tachykinins listed in Table I exist only in non-mammalian species. These are eledoisin (Erspamer and Anastasi, 1962), physalaemin (Erspamer et al., 1964), and kassinin (Anastasi et al., 1977). The tachykinins found in mammals are referred to as neurokinins, and include SP, neurokinin A (NKA; formerly known as substance K and neurokinin  $\alpha$ ; Kimura et al., 1983, 1984), and neurokinin B (NKB; formerly referred to as neuromedin K and neurokinin  $\beta$ ; Kangawa et al., 1983). Another endogenous neurokinin, neuropeptide K (NPK; Tatemoto et al., 1985), may or may not be an intermediate in NKA biosynthesis.

### Molecular Biology

The neurokinins, like other neuropeptides, are derived from larger protein precursors which are synthesized in the neuronal cell body. Post-translational modification of the precursor molecules occurs as they are transported to the nerve terminal. Here, the final products are stored until released. Studies have shown that two genes contain codons for the three mammalian neurokinins: the preprotachykinin A gene encodes for SP and NKA; and, the preprotachykinin B gene encodes for NKB (Kotani et al., 1986, Nawa et al., 1983, 1984). The preprotachykinin A gene produces three different preprotachykinin (PPT) mRNAs:  $\alpha$ ,  $\beta$  and  $\gamma$ .

Table I: Tachykinin and Analogue Amino Acid Sequences

<u>Tachykinin</u>	<u>MW</u>	<u>Abbreviation</u>	<u>Amino acid sequence</u> <sup>a</sup>
Substance P	1348	SP	Arg-Pro-Lys-Pro-Gln-Gln- - <u>Phe-Phe-Gly-Leu-Met-NH<sub>2</sub></u>
Neurokinin A	1133	NKA	His-Lys-Thr-Asp-Ser- - <u>Phe-Val-Gly-Leu-Met-NH<sub>2</sub></u>
Neurokinin B	1211	NKB	Asp-Met-His-Asp-Phe- - <u>Phe-Val-Gly-Leu-Met-NH<sub>2</sub></u>
Physalaemin	1266	PHY	pGlu-Ala-Asp-Pro-Asn-Lys- - <u>Phe-Tyr-Gly-Leu-Met-NH<sub>2</sub></u>
Eledoisin	1189	ELE	pGlu-Pro-Ser-Lys-Asp-Ala- - <u>Phe-Ile-Gly-Leu-Met-NH<sub>2</sub></u>
Kassinin	1335	KAS	Asp-Val-Pro-Lys-Ser-Asp-Gln- - <u>Phe-Val-Gly-Leu-Met-NH<sub>2</sub></u>
Dimethyl-C7	880	DiMe-C7	pGlu-Gln- <u>Phe-MePhe-Sar<sup>b</sup>-Leu-</u> - <u>Met-NH<sub>2</sub></u>
Senktide	842		Succinyl-Asp- <u>Phe-MePhe-Gly-</u> - <u>Leu-Met-NH<sub>2</sub></u>

<sup>a</sup> (common amino acids are underlined)

<sup>b</sup> Sar = N-methyl glycine

The  $\alpha$  PPT encodes for SP only, while the  $\beta$  and  $\gamma$  forms encode for both SP and NKA (Krause et al., 1987). Furthermore, it is now known that  $\beta$ -PPT also encodes for neuropeptide K (Tatemoto et al., 1985), which probably represents an elongated or pre-form of NKA (Martling et al., 1987; Deacon and Conlon, 1987). Precursor forms of SP (intermediates in proteolytic processing) also have been detected in rat brain (Kream et al., 1985). The PPT mRNA and tachykinin precursors have been found in human brain as well (Nyberg et al., 1985; Chesselet and Affolter, 1987).

### Release

Neurokinin release from nerve terminals may be mediated by a calcium and potassium dependent mechanism (Lindefors et al., 1985 a). This has been demonstrated in slice and synaptosomal preparations of hypothalamus, ventral mesencephalon, and substantia nigra (Iversen et al., 1976; Schenker et al., 1976; Jessel, 1978; Kronheim et al., 1980; Torrens et al., 1981). Electrically-stimulated release also has been demonstrated in brain and spinal cord (Otsuka and Konishi, 1976; Michelot et al., 1979; Akagi et al., 1980).

### Degradation

The degradation and inactivation of tachykinins are important considerations in the study of the pharmacology and physiology of these peptides. A number of peptidases have been postulated to be involved in the enzymatic cleavage and degradation of SP. These include: angiotensin-converting enzyme (Thiele et al., 1985), enkephalinase (endopeptidase 24.11; Matsas et al., 1984), dipeptidylaminopeptidase IV (Heymann and Mentlein, 1978), the so-called "substance P-degrading enzyme" (Lee et al., 1981; Probert and Hanley, 1987), "bacitracin-sensitive enzyme" (Horsthemke et al., 1984), and calpain (Hatanaka et al., 1985). Since many of these studies employed exogenous peptide, it has been suggested they may not accurately represent the activity of the peptidases in vivo (Mauborgne et al., 1987). Using superfused slice preparations, these authors have suggested that the endogenous SP released from the substantia nigra is primarily inactivated by enkephalinase and calpain (Mauborgne et al., 1987). Others have suggested the involve-

ment of enkephalinase in the physiological degradation of SP (Oblin et al., 1988). Indeed, recent studies indicate that enkephalinase hydrolyses NKA and NKB, as well as SP (Hooper and Turner, 1986; Turner and Hooper, 1987). However, the physiological relevance of these findings has yet to be determined.

### Neurokinin Antagonists

There have been a number of SP-related antagonists developed (Engberg et al., 1981; Folkers et al., 1981; Rosell and Folkers, 1982; Regoli, 1985; Escher et al., 1987; Regoli et al., 1987), and used to classify neurokinin binding sites (see below; Regoli et al., 1984; Jacoby et al., 1987; Murray et al., 1987; Vaught et al., 1987). However, several studies indicate that these compounds are neurotoxic, have local anesthetic properties, and actions not related to their antagonist activities, thus making them unsuitable for use as in-vivo competitive blockers (Hokfelt et al., 1981; Salt et al., 1982; Post et al., 1985; Freedman et al., 1986; Lembeck et al., 1986; Yusof and Coote, 1987; Dr. Peter J. Elliott, personal communication, 1986). Interestingly, recent studies have revealed that rats are uniquely susceptible to these non-specific neurotoxic actions (Post et al., 1987; Vaught and Scott, 1987). It has been suggested that D-trp substitution may underlie this neurotoxicity (Post and Paulsson, 1985; Vaught and Scott, 1987). The development of specific, non-neurotoxic, non-anesthetic neurokinin antagonists is necessary.

### Localization Within the CNS

Using immunocytochemical procedures and polyclonal antisera directed against SP, Ljungdahl et al. (1978 a,b) and Cuello and Kanazawa (1978), published the first major studies on the distribution of SP-like cell bodies and fibers in the adult rat CNS. These were followed by the studies of Inagaki et al. (1982) and Sakanaka et al. (1982) on the distribution of SP-like neurons in the pre-and post-natal rat brain. Although other reports preceded these (for example, Nilsson et al., 1974; Hokfelt et al., 1975), the aforementioned studies represent the first detailed mapping of SP-containing perikarya and fibers in the rat CNS. Accordingly, SP-like immunoreactive (SPLI) cell bodies have been localized in many brain regions, including: the nucleus accumbens, the septal area, the basal forebrain, caudate-putamen, bed nucleus of the stria terminalis, medial habenular nucleus, thalamus, zona incerta, amygdala, hippocampus, lateral hypothalamus, central gray, interpeduncular nucleus, dorsal raphe nucleus, superior colliculus, medullary raphe nuclei, medullary ventrolateral reticular formation, and, the spinal cord (Ljungdahl et al., 1978 a). SPLI fibers and terminals, furthermore, have been observed in most regions of the brain (Cuello and Kanazawa, 1978; Ljungdahl et al., 1978 a). Importantly, SPLI has been discerned in the midbrain raphe nuclei (Cuello and Kanazawa, 1978; Ljungdahl et al., 1978 a; Shults et al., 1984).

Since the discovery of the other mammalian neurokinins, namely, NKA and NKB, no one has attempted to thoroughly map the neurokinin-containing cell bodies within the CNS. Most of the recent work has focussed on discrete projection systems and specific areas. Warden and

Young (1988), however, recently mapped SP and NKB mRNAs in the rat CNS, and found that these mRNAs have distinct patterns of distribution.

The antibodies originally used to detect SPLI have been found to cross-react with the other neurokinins or their precursors (Dr. Tomas Hokfelt, personal communication, 1986; Brodin et al., 1986). Thus, with the demonstration that other neurokinins and their mRNAs exist in the mammalian CNS, the specificity for SP-like immunoreactivity of the early mapping studies has been questioned (Dr. Tomas Hokfelt, personal communication, 1986).

Biochemical procedures, such as radioimmunoassay (RIA), and high performance liquid chromatography (HPLC), have been used to examine the distributions and concentration of the neurokinins in the CNS. Since these techniques employ tissue extractions, antisera can be developed which recognize portions of the peptide strand inaccessible in vivo. Hence, the antibodies are more specific and less cross-reactive. A number of investigators have used these methods to determine the concentration of the neurokinins in distinct CNS structures (Brownstein et al., 1976; Minamino et al., 1984; Theodorsson-Norheim et al., 1984; Lindefors et al., 1985, 1986; Shults et al., 1985 b; Arai and Emson, 1986; Brodin et al., 1986; Nagashima et al., 1987; Diez-Guerra et al., 1988).

#### Binding Sites

Ligand-binding and in vitro bioassay studies have led to the identification of three distinct neurokinin recognition sites: NK-1 (SP-preferring), NK-2 (NKA-preferring), and NK-3 (NKB-preferring; Lee

et al., 1982, 1986; Regoli et al., 1984 a,b; Quirion, 1985; Henry, 1987). An effort has been made to correlate binding affinity with biological activity (Dion et al., 1987). In the discussion which follows, the characterization of peripheral neurokinin binding sites will be considered briefly, followed by a more detailed discussion of their properties in the CNS.

#### Peripheral Pharmacological Neurokinin Bioassays

The physiological properties of the neurokinins have been examined in various smooth muscle preparations, including the guinea pig ileum, trachea, and gallbladder; the rat vas deferens, duodenum, and portal vein; the rabbit pulmonary artery, heart, and mesenteric vein; the pig coronary artery; the dog carotid artery; and, the isolated human urinary bladder (Regoli et al., 1984 a,b,c; Mastrangelo et al., 1986; Osakada et al., 1986; Shook and Burks, 1986; Dion et al., 1987; Guard and Watson, 1987; Gulati et al., 1987; Munekata et al., 1987; Rovero and Pestellini 1987; Tousignant et al., 1987; Maggi et al., 1988). Some of the contractile effects of the neurokinins may be mediated through the release of other transmitters, such as acetylcholine (Holzer and Lembeck, 1980; Guard and Watson, 1987).

Based upon the relative potency of various agonists to contract smooth muscle, and their blockade by competitive antagonists, different muscles are thought to possess differing numbers or densities of neurokinin receptors. For example, in the isolated pig coronary artery the relative rank order of potency of the tachykinins is: SP - PHY > ELE > KAS > NKA > NKB, with SP being 9 and 90 times more potent than NKA and

NKB, respectively. This smooth muscle preparation appears to contain a high density of NK-1 sites, and can be used as an assay for NK-1 agonists (Gulati et al., 1987). The guinea pig ileum can be used as a bioassay for the NK-2 binding site (Shook and Burks, 1986). In this smooth muscle preparation, the relative rank order of potency for the force of contraction is: NKA > KAS > ELE >> PHY > SP, with NKA approximately 175 times more potent than SP. The contraction of the detrusor muscle of the human isolated urinary bladder also has been shown to be mediated predominantly by NK-2 sites (Maggi et al., 1988). The rat isolated portal vein bioassay has been demonstrated to be most sensitive to NKB (Mastrangelo et al., 1986; Dion et al., 1987). Recently, there has been an effort to correlate the effects of neurokinins in in vitro receptor bioassays with in vivo physiology (Kawahara and Yanai-hara, 1987; Maggi et al., 1987).

#### Characterization of CNS Neurokinin Binding Sites

The characteristics and localization of neurokinin binding sites in the CNS have been reviewed recently (Glowinski et al., 1987; Saffroy et al., 1988). The delineation of the neurokinin binding sites has relied on the use of  $^{125}\text{I}$ -Bolton-Hunter (BH) and  $^3\text{H}$ -labeled peptides, and analogues in membrane and synaptosomal preparations. In rat cortical synaptosomes, binding of  $^{125}\text{I}$ -BH-SP to NK-1 sites, and  $^{125}\text{I}$ -BH-ELE to NK-3 sites (Beaujouan et al., 1986; Bergstrom et al., 1987), is specific, temperature-dependent, saturable, and reversible, with  $K_d$ 's in the nanomolar range (Bergstrom et al., 1987; Viger et al., 1985). These authors report that Scatchard and Hill plots for each indicate a

single class of non-interacting binding sites. Competitive studies have revealed that SP and NKB inhibit binding of  $^{125}\text{I}$ -BH-SP and  $^{125}\text{I}$ -BH-ELE, respectively, in the nanomolar range (Glowinski et al., 1987).

It has been shown that the length of the peptide or analogue will affect its binding. For instance, longer fragments are more potent at NK-1 sites than shorter C-terminal fragments and analogues, such as DiMe-C7 and senktide (see Table 1), which are more potent at NK-3 binding sites (Cascieri and Liang, 1983; Torrens et al., 1984; Viger et al., 1985).  $^3\text{H}$ -NKB and  $^{125}\text{I}$ -BH-ELE have been reported to label identical sites (Bergstrom et al., 1987).

#### NK-2 Binding Sites in the CNS

NK-2 binding sites have been distinguished from NK-1 and NK-3 sites in peripheral tissues (Buck et al., 1984, 1986; Burcher et al., 1986). Their presence in the CNS has been suggested, chiefly through autoradiographic studies (Mantyh et al., 1984 b; Quirion and Dam, 1985; Shults et al., 1985 a; Buck et al., 1986). However, the presence of NK-2 recognition sites in the CNS has been questioned. Competition studies using rat cortical synaptosomes have shown that  $^{125}\text{I}$ -BH-NKA has a higher affinity than NKA for sites labeled with  $^{125}\text{I}$ -BH-ELE or  $^3\text{H}$ -NKB, suggesting that NK-3 sites are being labeled (Bergstrom et al., 1987; Glowinski et al., 1987; Saffroy et al., 1988). Autoradiographic analysis, furthermore, has indicated that the specific binding of  $^{125}\text{I}$ -NKA and  $^3\text{H}$ -NKA is very low, and that they label sites identical to those which bind  $^3\text{H}$ -SP or  $^{125}\text{I}$ -BH-SP. Likewise,  $^{125}\text{I}$ -BH-NKA,  $^3\text{H}$ -NKB and  $^{125}\text{I}$ -BH-ELE appear to label the same recognition sites (Buck et

al., 1986; Saffroy et al., 1988). Thus, the presence of NK-2 binding sites within the CNS needs to be examined more thoroughly.

#### Second-Messenger Coupling

There is some evidence that the neurokinins can stimulate phosphatidylinositol breakdown in the periphery and in the CNS (Watson and Downes, 1983; Mantyh et al., 1984 c; Watson, 1984; Torrens et al., 1986). There also is evidence that SP receptors are coupled to guanine nucleotide proteins (Sharma and Musacchio, 1987). However, details of events at the molecular level following neurokinin binding need to be more thoroughly examined.

#### Conformational Studies

Recently, a number of studies have been aimed at determining the three-dimensional structure of the neurokinins and related peptides and how they interact with their respective binding sites (Chassaing et al., 1986, 1987; Drapeau et al., 1987; Hashimoto et al., 1987; Ploux et al., 1987; Rovero and Pestellini, 1987; Lavielle et al., 1988). Through the construction of conformationally constrained analogues, it is hoped that selective and stable analogues and/or antagonists will be found. This approach has led to the development of a number of neurokinin analogues including: DiMe-C7 [(pGlu<sup>5</sup>, MePhe<sup>8</sup>, Sar<sup>9</sup>)SP(5-11)], originally developed as a metabolically stable analogue of SP (Sandberg et al., 1982); septide [(pGlu<sup>5</sup>, Pro<sup>9</sup>)SP(6-11)], a selective agonist for NK-1 binding sites (Laufer et al., 1986 a; Wormser et al., 1986), and senktide [(succ-Asp<sup>6</sup>, MePhe<sup>8</sup>)SP(6-11)], a selective agonist for NK-3

sites (Laufer et al., 1986 b; Wormser et al., 1986). It has been shown that compounds with an N-methyl group at position 8, such as DiMe-C7 and senktide, are associated with a dramatic loss of potency at NK-1 sites and an increase in potency at NK-3 sites (Drapeau et al., 1987; Lavielle et al., 1986; Ploux et al., 1987). Ploux et al. (1987) and Lavielle et al. (1988) have suggested that the  $\alpha$  helical core which the neurokinins adopt when dissolved in methanol, may be related to their bioactive conformation in vivo. After these peptides bind to their receptor, they appear to form similar three-dimensional structures. However, the non-homologous N-terminal sequences may influence the orientation of the C-terminal Gly-Leu-Met-NH<sub>2</sub> trimer such that it binds to its appropriate neurokinin receptor.

#### Distribution of CNS Neurokinin Binding Sites

The localization and distribution of neurokinin binding sites has been extensively studied using autoradiography (Wolf et al., 1983; Mantyh et al., 1984 a,b; Shults et al., 1984, 1985 a,b; Buck et al., 1986; Danks et al., 1986; Beaujouan et al., 1986; Saffroy et al., 1988). These studies have indicated that neurokinin binding sites are present, to varying degrees, throughout the CNS. Although neurokinin binding sites tend to be rather ubiquitous, certain differences in distribution can be found. For instance, high densities of NK-1 sites have been found in the olfactory bulb; the prefrontal cortex; the hippocampus; the anterior cortical nucleus of the amygdala; the septal area; the medial habenular nucleus; the dorsal raphe; the superior colliculus; cerebellar lobules 9 and 10; the dorsal tegmental nucleus of

Gudden; the locus ceruleus; and, the dorsal motor nucleus of the vagus (Mantyh et al., 1984 a; Beaujouan et al., 1986; Buck et al., 1986; Saffroy et al., 1988). The density of NK-3 binding sites is highest in the cerebral cortex; the basolateral amygdaloid nucleus (anterior); the zona incerta; magnocellular part of the paraventricular hypothalamic nucleus; the perifornical and supraoptic hypothalamic nuclei; the interpeduncular nucleus; the ventral tegmental area; and, the nucleus of the solitary tract (Beaujouan et al., 1986; Buck et al., 1986; Danks et al., 1986; Saffroy et al., 1988).

With respect to the midbrain raphe, Saffroy et al. (1988) found a greater number of NK-1 than NK-3 sites in the DR, but about the same number (although less than in the DR) of both in the MR. Others, however, have found more  $^{125}\text{I}$ -BH-SP binding sites than  $^{125}\text{I}$ -BH-ELE sites in the MR (Buck et al., 1986).

#### Neurokinin Transmitter/Binding Site Mismatches

In certain brain regions, mismatches occur between the localization of a peptide and its receptor or binding site (Kuhar et al., 1985; Herkenham, 1987). For the neurokinins, the most illustrative and most often cited example of a brain structure which displays this phenomenon is the substantia nigra. This area contains the highest concentrations of SP and NKA in the brain (Kanazawa et al., 1984; Brodin et al., 1986) with negligible amounts of NKB (Kanazawa et al., 1984; Arai and Emson, 1986). Behavioral, electrophysiological, and biochemical effects have been observed following SP and NKA injections into the pars compacta and pars reticulata of the substantia nigra (Cheramy et al., 1977;

Kelley and Iversen, 1978 a,b, 1979; Pinnock and Dray, 1982; Innis et al., 1985). However, NK-1 sites have not been localized in this region (Mantyh et al., 1984 a; Shults et al., 1984; Beaujouan et al., 1986; Buck et al., 1986; Danks et al., 1986; Saffroy et al., 1988). Only a small number of NK-2 and NK-3 sites, furthermore, have been discerned in the substantia nigra (Mantyh et al., 1984 b; Quirion and Dam, 1985; Shults et al., 1985 a; Beaujouan et al., 1986; Buck et al., 1986; Danks et al., 1986; Saffroy et al., 1988).

Explanations for the peptide concentration/receptor density mismatches include: 1) technical problems associated with autoradiographic techniques; 2) low affinity but physiologically functional binding sites, which are not detectable autoradiographically; 3) internalization of receptors; and, 4) endogenous ligands occupying the receptors thus preventing the binding of radiolabeled compounds (Herkenham et al., 1987). Anatomical substance/receptor mismatch suggests that the substance has no function in that particular brain region, or that it has some undetectable neurohumoral or maintenance function. Behavioral, electrophysiological, and biochemical studies, nevertheless, may indicate a function, but the mechanism is unclear. Neurokinin/binding site mismatches in the substantia nigra remain an anomaly which, when resolved, will surely add to our knowledge of neurotransmitter/receptor interactions.

#### Recent Advances in Neurokinin Receptor Analysis

Some recent biochemical studies have led to the solubilization and characterization of a specific SP binding protein (Nakata et al.,

1988). Molecular biologists have been able to successfully isolate cDNA clones for bovine NKA and SP receptor mRNAs, and to express functional receptors in a Xenopus oocyte system (Masu et al., 1987; Buck et al., 1988). These unique and exciting approaches will allow the characterization of neurokinin receptors, and provide models for examining ligand-receptor coupling, second messenger systems, and receptor-membrane interactions.

### Electrophysiology

The electrophysiology of the neurokinins has not been thoroughly explored. Until recently, most reports have dealt primarily with the effects of SP. A number of studies have reported that iontophoretic application or micropressure injection of SP and other neurokinins produce excitatory effects. For example, SP induces a slow depolarization probably by decreasing potassium conductance (Dun, 1985). SP also has an excitatory effect on neurons in the hypothalamic-preoptic area (Shibota et al., 1988), locus ceruleus (Olpe et al., 1987 b), caudal trigeminal nucleus (Salt and Hill, 1980), chick sympathetic ganglia (Ramirez and Chiappinelli, 1987), dorsal root ganglia (Roberts and Wright, 1981), and interpeduncular nucleus (Sastry, 1978). Both SP and NKA have been shown to excite cells in the substantia nigra (Pinnock and Dray, 1982; Innis et al., 1985) and spinal cord (Henry, 1976; Henry and Salter, 1987). Excitatory and inhibitory responses to SP have been observed in the spinal cord (Belcher and Ryall, 1977) and dorsolateral septum (Nayar et al., 1987). SP-induced depression of neuronal activity in the rat olfactory bulb is thought to be mediated by GABA inter-

neurons (Olpe et al., 1987 a). Although the tachykinins excite spinal cord neurons, physalaemin has been reported to enhance the depressant effect of adenosine triphosphate (Salter and Henry, 1987).

Problems encountered in electrophysiological studies with tachykinins include their degradation within and adherence to the glass micropipettes used for iontophoresis (Gozlan et al., 1977; Guyenet et al., 1979; Lamour et al., 1983). Micropressure application (Palmer et al., 1986) does not require ejection or retaining currents, and may be a more suitable method for the delivery of these peptides during electrophysiological experiments (Dray et al., 1983).

#### Behavioral Effects of Neurokinins

The majority of studies concerning the behavioral effects of the neurokinins have been focused on the A9 and A10 DA cell groups in the ventral mesencephalon. SP has been shown to activate DA systems which originate in this region (Davies and Dray, 1976; Cheramy et al., 1977). The initial behavioral studies found that bilateral infusions of SP into the ventral tegmental area (VTA) increased LMA and enhanced exploratory behavior (Stinus et al., 1978). These authors also reported that the behavioral arousal induced by the systemic administration of d-amphetamine was potentiated by a prior infusion of SP into the VTA. Subsequent studies suggested that DA neurons mediate SP-induced behavioral arousal. Infusions of haloperidol into and 6-hydroxydopamine (6-OHDA) lesions of the nucleus accumbens block the hyperactivity produced by intra-VTA SP (Kelley et al., 1979). SP infusions into the substantia nigra (SN) have been observed to stimulate LMA and to produce

grooming, stereotyped rearing, and sniffing (Kelley and Iversen, 1978 a,b, 1979), which could be blocked by 6-OHDA lesions in the caudate nucleus (Kelley and Iversen, 1979). Thus, SP infusions into the VTA markedly increase LMA and exploratory behavior, whereas SP infusions into the SN produced stereotypic behaviors (Stinus et al., 1978; Kelley and Iversen, 1979). The behavioral effects elicited by repeated injections of SP into the VTA do not change in either their appearance or intensity, whereas those observed following repeated SN infusions change from increased rearing to increased sniffing and grooming (Stinus et al., 1978; Kelley and Iversen, 1979).

Unilateral intranigral SP infusions increase the activity of nigrostriatal DA neurons (Davies and Dray, 1976), increase DA turnover in the ipsilateral striatum (Cheramy et al., 1977; Baruch et al., 1987), and produce contralateral turning (James and Starr, 1977; Olpe and Koella, 1977). James and Starr (1979) later demonstrated that infusions of SP into the pars reticulata of the SN produce contralateral circling and increased DA turnover in the ipsilateral neostriatum, whereas infusions into the pars compacta induce ipsilateral circling, decreased DA turnover, and increased 5-HT turnover in the ipsilateral neostriatum.

The behavioral effects observed after SP administration are short lived (5-15 min). DiMe-C7, which was developed as a metabolically stable SP analogue (Sandberg et al., 1981), produces behavioral effects of a greater duration (30-60 min; Eison et al., 1982 a,b). Subsequently, NKA-immunoreactive fibers and receptors were demonstrated in the VTA, and intra-VTA NKA injections were shown to produce behavioral

effects after doses approximately ten times lower than the minimally effective SP dose (Kalivas et al., 1985).

Elliott and Iversen (1986) have characterized the behavioral effects of the tachykinins after their bilateral administration into the lateral ventricles and the VTA. They reported that ELE and DiMe-C7 were more potent than SP at increasing LMA. These authors also found that the ability of the various peptides to produce wet dog shakes and grooming varied as a function of the injection site. DiMe-C7 and ELE were more potent in producing wet dog shakes when administered into the ventricles, whereas DiMe-C7 and SP were more potent when administered into the VTA (Elliott and Iversen, 1986). They found that grooming was stimulated more effectively by SP and physalaemin by either route of administration. These studies illustrate the importance of employing several different tachykinins in order to delineate the function of the neurokinin receptors in a given brain region.

#### Behavioral Effects of Substance P Fragments

Stewart and colleagues have suggested that the N-terminal and C-terminal portions of the SP molecule produce different behavioral effects (Stewart et al., 1982; Hall and Stewart, 1983). Others have advanced this notion as well (Pinnock et al., 1983). The N- and C-terminal fragments can produce opposite effects on grooming, hot plate latencies, and stress-induced analgesia (Hall et al., 1987). These differential effects may be due to the processing of the SP molecule allowing the cleavage products to bind, perhaps, to different receptors (Hall and Stewart, 1983). These findings provide a means to explain

the diversity of effects induced by SP. On the other hand, as previously discussed, the short C-terminal fragments have a higher affinity for the NK-3 binding site (Cascieri and Liang, 1983; Torrens et al., 1984; Viger et al., 1985). Thus, the SP fragments may bind to the different receptors and thereby produce different functional changes. Conformational studies also suggest that the entire SP molecule is necessary for biological activity (Ploux et al., 1987; Lavielle et al., 1988). More work is needed to elucidate the physiological role of SP fragments in vivo.

#### Avoidance Learning

SP has been shown to induce an impairment in avoidance learning by rats after its injection into the substantia nigra or amygdala, but a facilitation in learning when injected into the lateral hypothalamus (Huston and Staubli, 1978, 1979). SP infusions into the nucleus basalis magnocellularis have been found to facilitate retention of a step-up avoidance task (Kafetzopoulos et al., 1986). The N-terminal fragment of SP, SP1-4, has been reported to enhance avoidance retention when injected into the lateral ventricles (i.c.v.) of mice, but similar infusions of SP or its C-terminal portion, SP7-11, are without effect (Pellemounter et al., 1986). SP also has been found to facilitate the acquisition of an appetitively motivated learning task (Schlesinger et al., 1986).

### Reinforcing Properties

Staubli and Huston (1985) have suggested that SP may be involved in rewarding processes. Infusions of SP into the lateral hypothalamus (LH) of rats produce positively reinforcing effects in a conditioned place preference paradigm (Holzhauer-Oitzl et al., 1987). The positively reinforcing properties of SP injected into the LH may be due to a local vasodilatory action (Klugman et al., 1980). Others have reported that intra-LH infusions of SP leads to a reduction in operant responding for electrical stimulation of the LH (Goldstein and Malick, 1977), and that intra-cortical or intra-ventricular infusions of SP decrease self-stimulation of the prefrontal cortex (Mora and Ferrer, 1984). It also has been reported that injections of DiMe-C7 into the lateral ventricles may produce aversive effects (Elliott and Iversen, 1983), whereas injections of DiMe-C7 into the MR may produce rewarding effects (Lee, J. et al., 1987). The reinforcing properties of SP and DiMe-C7 need to be further examined in order to determine which neurokinin receptor and which site(s) is (are) involved.

### Spinally-Mediated Behavioral Effects

The effects of intrathecally administered SP and other tachykinins on nociception (Yashpal and Henry 1982, 1983, 1984; Cridland and Henry 1986, 1988; Henry and Salter, 1987; Sawynok and Nance, 1987) and cardiovascular function (Couture et al., 1985, 1987, 1988; Delbro, 1987; Hassessian et al., 1987; Helke et al., 1987) have been thoroughly studied. Behavioral responses following intrathecal and intracisternal administration also have been observed. In mice, intrathecal ad-

ministration of SP and ELE induces biting and scratching (Hylden and Wilcox, 1981; Rackham et al., 1981; Share and Rackham, 1981). More recently, Stoessl and colleagues (1987 a,b) demonstrated that intracisternal (or subcutaneous) administration of senktide induces wet dog shakes and forepaw treading in both mice and rats, presumably by a serotonergic mechanism.

### Sexual Behavior

SP also has been implicated in sexual behavior. Infusions of SP into the midbrain central gray has been observed to facilitate lordosis (Dornan et al., 1987). Anatomically, SP has been localized in the bed nucleus of the stria terminalis, a region known for its role in sexually differentiated brain function (Valcourt and Sachs, 1979; Malsbury and McKay, 1987). Also, infusions of SP into the lateral ventricles have been reported to inhibit ovulation in rats (Potargowicz and Traczyk, 1987).

### Neurokinin - 5-HT Interactions in the CNS

The behavioral and neurochemical effects of neurokinin administration suggest that these neuropeptide and 5-HT systems interact. Neurokinin/5-HT interactions have been deduced by observing the effects of drugs which alter 5-HT transmission. The intrathecal administration of SP in mice induces hind limb scratching (similar to 5-HT), and the effect of intrathecally administered 5-HT can be blocked by an SP antagonist (Fasmer and Post, 1983; Fasmer et al., 1983; Hylden and Wilcox, 1981). Ogren et al. (1985) have suggested that SP may modulate 5-

hydroxytryptophan-induced head twitching. It has been demonstrated that i.c.v. administration of 5,7-DHT potentiates the responses to intrathecally applied SP (Eide et al., 1988) and decreases SP content and immunoreactivity in the medulla and spinal cord (Singer et al., 1979; Towle et al., 1986; Pare et al., 1987). Using mice and rats, Stoessl and colleagues (1987 a,b) have demonstrated that the intracisternal or subcutaneous administration of senktide induces a "5-HT syndrome" consisting of head twitches, forepaw treading, hind limb splaying, and wet dog shakes, which can be blocked by 5-HT<sub>2</sub> antagonists. In addition, it has been shown that the dose-dependent hyperactivity produced by intra-MR infusions of DiMe-C7 can be prevented by prior administration of PCPA or intra-MR 5,7-DHT administration (Paris and Lorens, 1987 a,b). The 5-HT<sub>3</sub> antagonist, GR38032F, furthermore, has been demonstrated to attenuate both the locomotor hyperactivity and the increase in DA turnover in the nucleus accumbens induced by intra-VTA administration of DiMe-C7 (Hagan et al., 1987).

Reisine et al. (1982) have reported that SP applied to the substantia nigra in the cat releases 5-HT in the contralateral caudate nucleus. Similarly, SP infusions into the MR increase 5-HT turnover in the hippocampus (Forchetti and Meek, 1982). Recently, it has been demonstrated that both SP and NKB stimulate basal and potassium-evoked 5-HT release in rat frontal cortex slices, presumably by distinct receptors (Solti and Bartfai, 1987).

Chronic treatment with the antidepressant drugs, imipramine and zimelidine, increases SP concentration in the dorsal and ventral spinal cord (Iverfeldt et al., 1986). Imipramine, but not zimelidine, also

increases potassium-stimulated SP release in a spinal cord-slice preparation. This is thought to be mediated by 5-HT receptors (Iverfeldt et al., 1986). In contrast to imipramine, chronic treatment (14 days) with the selective 5-HT reuptake inhibitors, zimelidine and alaprocolate, has been reported to increase SP-like immunoreactivity (SPLI) in the ventral spinal cord, and both SPLI and NKA/NKB-like immunoreactivity in the periaqueductal gray (Brodin et al., 1985, 1987). Repeated administration of imipramine leads to decreases in SPLI and NKA/NKB-like immunoreactivity in the frontal cortex, whereas alaprocolate produces opposite effects (Brodin et al., 1987). Cocaine has been reported to increase SPLI in the DR and to decrease pontomedullary 5-HT concentrations (Pradhan et al., 1981). 5-Hydroxytryptophan administration has been observed to decrease SPLI in the DR (Pradhan et al., 1981). Thus, the interactions between 5-HT and the neurokinins are diverse and clearly site-dependent. One area which deserves more study is the electrophysiological relationship between these two neurotransmitters.

### Conclusions

Since von Euler and Gaddum's (1931) initial discovery of SP, a great deal of information has been generated concerning the anatomy, physiology, biochemistry, and molecular biology of the neurokinins in both the CNS and periphery. Three distinct binding sites for the neurokinins have been identified which has led to the development of metabolically stable selective agonists. A variety of behavioral effects have been observed following neurokinin administration into the CNS. However, specific, non-toxic antagonists are required in order to

better characterize the functions of the distinct neurokinin recognition sites. A more thorough electrophysiological examination of neurokinin effects in the CNS is necessary as well. An anatomical analysis of the distribution of neurokinin cell bodies and neuronal pathways likewise is needed.

This dissertation project was designed to elucidate the origin and function of the neurokinin projections to the midbrain raphe. The objectives were: 1) the differentiation of the behavioral effects produced by the distinct neurokinin agonists; 2) to determine whether the neurokinin-induced behavioral effects depended on the integrity of 5-HT neurons; 3) to determine the origin of the neurokinin innervation of the raphe; and, 4) to demonstrate the functional significance of MR-neurokinin-induced behavioral arousal.

## CHAPTER III

### METHODS

#### A. Behavioral Studies

##### Animals

Male Sprague-Dawley rats (Sasco-King, Orange, WI), 90-120 days old and 275-325 g at the time of surgery, were used. The animals were housed individually in an illumination (12 hr light-dark cycle, lights on at 07:00 h), temperature ( $22 \pm 2^{\circ}\text{C}$ ), and humidity ( $50 \pm 5\%$ ) controlled facility. Food and water were available ad libitum. Following their arrival, the animals were acclimated to their new environment for at least one week prior to surgical intervention.

##### Cannula Implantation

The rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.), and treated with atropine sulfate (0.4 mg/kg, i.m., in 0.9% saline) and sodium ampicillin (50 mg/kg, i.m., in 0.9% saline). The rats were secured in a Kopf stereotaxic frame with the top of the incisor bar positioned 3.3 mm below the interaural plane according to the atlas of Paxinos and Watson (1986). Thimersol (1:1000), an anti-septic, was applied topically to the scalp. An incision was made, the skull exposed, and a craniotomy performed. A sterile stainless steel guide cannula (0.46 mm, o.d. and 0.25 mm, i.d.; Plastic Products,

Roanoke, VA) was lowered at an angle 25° lateral to the midsagittal plane, 3.2 mm lateral, 7.8 mm ventral and 7.5 mm caudal from bregma. The guide cannula contained a sterile stainless steel stylet (0.23 mm, o.d.) which extended 0.5 mm beyond its tip. The cannula was secured to the skull by means of acrylic cement and stainless steel screws. The wound margin was infiltrated with 2.0% lidocaine hydrochloride to minimize post-operative pain. The wound was closed with clips, and 1.0% chloramphenicol ointment, an antibiotic, was applied topically around the wound. Post-operatively the rats were kept warm on a heating pad until they regained their righting reflex. All animals were allowed to recover for at least one week prior to the initiation of behavioral testing.

#### Neurotoxin Lesions

The rats were prepared for surgery as described above. Briefly, the subjects were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and pretreated with the norepinephrine (NE) and dopamine (DA) reuptake inhibitor, nomifensine maleate (15 mg/kg/2.0 ml 0.9% saline, i.p.), 30-40 min prior to the neurotoxin infusion. One group of rats (n = 11) was first implanted with intra-MR cannulae. They then received an infusion of 5,7-DHT (8.0 µg, as the base, in 2.0 µl of 0.1% ascorbate in 0.9% saline) directly through the cannula. The drug was infused by a microsyringe pump (Model CMA/100, Carnegie Medicin AB, Solna, Sweden) over 10 min (0.2 µl/min). The internal cannula was left in situ an additional 3-5 min to allow adequate spread of the solution away from the tip. The stylet then was replaced and the animal allowed

to recover. In addition, two small burr holes were drilled in the skull at the point of entry for the medial forebrain bundle (MFB) injections (see below).

The second group of rats ( $n = 11$ ) received a bilateral infusion of 5,7-DHT ( $8.0 \mu\text{g}$ , as the base, in  $3.0 \mu\text{l}/\text{side}$  in the ascorbate vehicle) into the MFB via 28 gauge stainless steel tubing. Using bregma ( $\beta$ ) as the reference point, the coordinates were: anteroposterior,  $\beta - 3.8 \text{ mm}$ ; mediolateral,  $\beta \pm 1.7 \text{ mm}$ ; dorsoventral,  $\beta - 8.7 \text{ mm}$ . The drug was infused over 10 min ( $0.3 \mu\text{l}/\text{min}$ ), and the injection cannula was left in situ an additional 3-5 min to allow adequate spread of drug. Immediately following the infusion of 5,7-DHT, an intra-MR cannula was implanted.

Two separate groups of control rats were prepared. One group ( $n = 5$ ) was implanted with MR cannulae, then infused with  $2.0 \mu\text{l}$  of ascorbate vehicle in a manner identical to the MR 5,7-DHT lesion group. The second group of controls ( $n = 5$ ) received a bilateral infusion of ascorbate vehicle ( $3.0 \mu\text{l}/\text{side}$ ) into the MFB, as described above, prior to MR cannula implantation.

#### Apparatus

Locomotor activity measures were obtained using darkened, enclosed, cylindrical photocell chambers (46 cm diam x 42 cm high; LVE Model #PAC-001) with wire mesh floors ( $n = 10$ ). Activity counts were generated by interruption of any one of six photocell beams located at the base of the chamber and recorded on a digital counter.

Open field activity was observed using a plywood arena (100 x 100 cm) with 40 cm high unpainted walls. Illumination was provided by a 40 W bulb centered 1.0 m above the floor. The floor was painted white and divided by thin black lines into 25 equal squares (20 x 20 cm). The floor of the open field was elevated 7.5 cm above the floor of the testing room. Equidistantly spaced holes (3.5 cm diameter) were located in the four corner squares of the central nine squares to permit measurement of head pokes. A head poke was defined as the lowering of the animal's snout into a hole. The space directly below the arena (visible through the holes) was illuminated with a 25 cm, 20 W fluorescent tube. Each animal's behavior was videotaped for subsequent analysis.

#### Handling and Habituation

The rats were handled daily beginning 7-10 days after surgery. After 2-3 days of handling, habituation to the photocell chambers was initiated. Each rat was placed individually into an apparatus. Thirty min later, the animal was removed, gently wrapped in a towel for 30-60 sec, then returned to the chamber for 30 min. This procedure was repeated for five consecutive days (Days 1-5) in order to adapt each rat to the injection procedure. All behavioral testing was conducted between 09:00-14:00 h, and each rat was always tested in the same photocell cage.

For the open field experiments, the rats were handled daily beginning 7-10 days after surgery. For the studies which were conducted in a novel open field, the rats were removed from their home

cage and adapted for 3 days to the injection procedures as described above. Later, on each of these 3 days, each rat was handled for 1-2 min to further accustom the animal to the experimenter. The rats were exposed to the open field only once, on the day of drug testing. For the studies which involved habituation to the open field chamber, each rat was removed from its home cage, gently wrapped in a towel as described above, then placed in the center of the open field arena. The rat was allowed to explore the arena for 30 min, after which time it was removed and returned to its home cage. This procedure was repeated for five consecutive days. Drug effects were examined on the sixth day. The chamber was thoroughly cleaned with warm water and dried after removal of each rat. All testing was conducted between 09:00-14:00. Each rat's behavior was videotaped for subsequent analysis. The following behaviors were quantified: the number of wall and center squares entered, rears (rat stands on hind limbs and sniffs), and head pokes.

#### Drug Testing

Drug testing for the LMA experiments began on the day (Day 6) immediately following the last day of habituation. Vehicle or different drug doses were administered every other day, in a randomized fashion. The animals were placed in the chambers daily for 60 min, but no data were collected on non-drug days. Total activity counts for the 30 min of post-drug testing were recorded.

The injection procedure consisted of removing the animal after the 30 min pre-drug habituation period, and gently wrapping it in a

towel. The stylet was removed and replaced with the internal cannula which was connected, via polyethylene tubing, to a 25  $\mu$ l Hamilton syringe driven by a microsyringe pump (Model CMA/100, Carnegie Medicin AB, Solna, Sweden). The drugs and vehicle were administered at a rate of 1.0  $\mu$ l/min. The volume of all infusions was 1.0  $\mu$ l and the total time for each infusion was 1.0 min. Upon completion of the injection, the internal cannula was left in situ for an additional 30 sec, after which time it was removed and the stylet replaced.

For the open field studies, the rats were removed from their home cages, gently wrapped in a towel, and infused with a neurokinin or vehicle as described above. Following completion of the drug infusion, the rat was immediately placed in the center of the open field arena, and its behavior videotaped for 24 or 30 min. The rat then was returned to its home cage.

#### Drugs

DiMe-C7 [(pGlu<sup>5</sup>, MePhe<sup>8</sup>, Sar<sup>9</sup>)substance P 5-11], neurokinin A (NKA), substance P (SP), neurokinin B (NKB), eleoisin (ELE), kassinin (KAS), physalaemin (PHY) (Peninsula Laboratories, Belmont, CA), and senktide [(succinyl-Asp<sup>6</sup>, MePhe<sup>8</sup>)substance P 6-11] (Bachem, Torrance, CA) were prepared according to the method of Stewart (1982). The lyophilized peptides were dissolved in 0.01 M acetic acid and diluted to a stock concentration with phosphate buffered saline (PBS; pH 7.4). The stock solutions (DiMe-C7: 6.0  $\mu$ g/ $\mu$ l; NKA: 6.0  $\mu$ g/ $\mu$ l; SP: 3.3  $\mu$ g/ $\mu$ l; senktide: 2.0  $\mu$ g/ $\mu$ l; ELE: 3.3  $\mu$ g/ $\mu$ l; KAS: 3.5  $\mu$ g/ $\mu$ l; PHY: 6.0  $\mu$ g/ $\mu$ l) then were aliquoted and kept frozen (-20°C). On each drug day, an

aliquot was thawed and diluted with vehicle (PBS with 0.01 M acetic acid added, pH 7.2).

NKB first was dissolved in 88% formic acid, then diluted to a stock concentration ( $4.4 \mu\text{g}/\mu\text{l}$ ) with phosphate buffer (pH 11.8). The stock solution was aliquoted and kept frozen ( $-20^{\circ}\text{C}$ ). On each drug day, an aliquot was thawed and diluted with vehicle (PBS with 88% formic acid added, pH 3.8).

All peptide solutions were kept dark and cold until used. Care was taken throughout to avoid the use of glass or polystyrene which will bind the peptide and dramatically alter the amount delivered (Stewart, 1982, 1983).

Peptide doses were calculated as moles/ $\mu\text{l}$ . In order to account for differences in purity which can affect the desired drug concentration (Stewart, 1982), analyses were obtained from each manufacturer for each batch of peptide received. These can be found in Appendix I. The purity of all peptides was greater than 80%. The molar concentrations used took into consideration the purity of the peptide, and stock solutions were appropriately prepared. These calculations also can be found in Appendix I.

Sodium pentobarbital was obtained from Butler (Columbus, OH); thimersol (Merthiolate<sup>R</sup>) from Eli Lilly (Indianapolis, IN); lidocaine hydrochloride (Xylocaine<sup>R</sup>) from Astra (Worcester, MA); chloramphenicol (Chloromycetin<sup>R</sup> 1.0% ophthalmic ointment) from Parke-Davis (Morris Plains, NJ); atropine sulfate, sodium ampicillin and 5,7-dihydroxytryptamine-creatinine sulfate were purchased from Sigma (St. Louis, MO).

### Neurochemical Measurements

The animals were sacrificed by decapitation, their brains removed, and dissected on a brass plate over ice. The amygdala, hippocampus, hypothalamus, medial frontal cortex, nucleus accumbens and striatum were obtained using a modification of the method described by Heffner et al., (1980). The tissue was immediately frozen on dry ice, then placed in microcentrifuge tubes and stored at  $-80^{\circ}\text{C}$ . Tissue levels of 5-HT, 5-hydroxyindoleacetic acid (5-HIAA), DA, dihydroxyphenylacetic acid (DOPAC), norepinephrine (NE), and homovanillic acid (HVA) were determined within 1-2 weeks in each of the brain areas by high performance liquid chromatography (HPLC) with electrochemical detection.

The frozen brain samples were weighed and sonicated in 0.1 N perchloric acid (PCA) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA), 2.0 mM sodium metabisulfite (an antioxidant), 50 ng of the internal standard, N-methyl-5-hydroxytryptamine (NM5-HT), and/or 10 ng of the internal standard, 3,4-hydroxybenzylamine (DHBA). The homogenate then was centrifuged at  $15,000 \times g$  for 10 min.

For brain regions which did not require concentration of the amines and metabolites (nucleus accumbens and striatum), the resulting supernatant was aspirated, added to minivials, then placed into a Beckman 504 autosampler at room temperature. The mobile phase consisted of 0.15 M monochloroacetic acid, 0.8 mM EDTA, 18% methanol and 100 mg/L of octanysulfonic acid (OSA). The retention times for these conditions were: DA, 5.2 min; DOPAC, 6.5 min; 5-HIAA, 9.9 min; 5-HT, 11.2 min; NM5-HT, 13.3 min; HVA, 15.2 min.

For brain regions which required concentration of the amines and their metabolites (amygdala, hippocampus and medial frontal cortex), an alumina extraction was performed. The supernatant (100  $\mu$ l) from the initial centrifugation was added to 50 mg activated alumina. To this suspension, 50  $\mu$ l of 3.0 M Tris-HCl (pH 8.4) was added and shaken vigorously for 10 min. Then, 40  $\mu$ l 0.1 N PCA was added and the suspension centrifuged for 2.0 min. The supernatant was aspirated, added to minivials, and loaded into the autosampler at room temperature. The mobile phase consisted of 0.15 M monochloroacetic acid, 0.8 mM EDTA, 20% methanol, and 10 mg/L of OSA. The retention times for the substances contained in the supernatant were: 5-HT, 5.7 min; NM5-HT, 6.9 min; 5-HIAA, 9.9 min.

The alumina pellet from the above extraction was washed twice in 200  $\mu$ l of 2.0 mM Tris-HCl containing 2.0 mM sodium metabisulfite, vortexed, then centrifuged for 5.0 sec. Following the second wash, 50  $\mu$ l of 0.2 N PCA containing 2.0 mM sodium metabisulphite was added to the alumina pellet. The samples then were shaken vigorously and centrifuged for 5.0 sec. The resulting supernatant was aspirated, added to minivials and loaded into the autosampler. The mobile phase consisted of 0.15 M monochloroacetic acid, 0.8 mM EDTA, 15% methanol, and 750 mg/L of OSA. The retention times for the substances contained in the supernatant were: NE, 5.1 min; DOPAC, 6.4 min; DHBA, 8.6 min; and DA, 12.4 min.

All mobile phases were filtered (0.2  $\mu$ m), degassed under a vacuum, and delivered via a Beckman solvent delivery system (Model 110B). The flow rate for all separations was 0.9 ml/min. Aliquots of tissue

samples or standards were injected (20  $\mu$ l) onto a reverse-phase column (5.0  $\mu$ m 4.6 mm x 15 cm Beckman column. Rainin Co., Woburn, MA) equipped with a guard column [Bioanalytical Systems (BAS), West Lafayette, IN]. The effluent from the analytical column was passed through a BAS LC-4 electrochemical detector using a glassy carbon electrode with an applied potential of +0.74 V. The electrical output was quantified using a Beckman integrator (Model 427; Spectra-Physics, Santa Clara, CA).

### Sacrifice

The animals used in the 5,7-DHT lesion study were sacrificed by decapitation. Rats used in all other experiments were injected with an overdose of sodium pentobarbital (90 mg/kg, i.p.) and perfused transcardially with isotonic saline (100 ml) followed by 10% buffered formalin (100 ml). The brain stems were stored in formalin for at least two weeks prior to histological analysis.

### Histology

Frozen coronal sections (50  $\mu$ m) through the cannula placements were cut on a sliding microtome. Every fourth section was float mounted from cold phosphate buffered saline (PBS) onto gelatin-chrome alum coated "subbed" slides, and subsequently stained with cresyl violet acetate. A representative cannula placement is depicted in Fig. 1 (p.62).

### Statistical Analysis

All data were subjected to the appropriate analysis of variance (ANOVA). Post-hoc comparisons of means were made with Duncan's multiple range test (Winer, 1971). For the repeated measures analyses, post-hoc comparisons were made only on the factors which were determined by the ANOVA to be significant at the 0.05 probability level. Data from the NKB injection study were analyzed with a Student's t-test (two-tailed). The neurochemical data were analyzed by ANOVA or Student's t-test where appropriate. In order to compare the relative potencies of the various tachykinins for eliciting hyperactivity, the data were analyzed by means of a regression analysis of the log-doses of the peptide versus the total activity. Tests for parallelism were performed on the various regression lines (Kenakin, 1987; Tallarida and Jacob, 1979) utilizing the computer programs of Tallarida and Murray (1979).

### B. Anatomical Studies

#### Animals

Rats were obtained and housed as described above (p. 44).

## Retrograde Tracing

### Iontophoresis

Glass micropipettes with a tip diameter of 15-20  $\mu\text{m}$  were made from glass tubing (100  $\mu\text{m}$  outside diameter) using a Narashige electrode puller.

The rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.); treated with sodium ampicillin (50 mg/kg, i.m.) and atropine sulfate (0.4 mg/kg, i.m.); and, placed in a Kopf stereotaxic apparatus. An incision was made and a craniotomy performed. Fluoro-Gold (FG) was iontophoresed into the MR, with the aid of a dissecting microscope, using the following coordinates: anteroposterior, bregma ( $\beta$ ) - 7.5 mm; mediolateral,  $\beta \pm 3.4$  mm; dorsoventral,  $\beta - 7.7$  mm. These were determined empirically by iontophoresing the dye, fast green. A Midguard constant current generator (Harvard Apparatus Inc., South Natick, MA) supplied 7.0 sec pulses of a 6.0  $\mu\text{A}$  positive current every 7.0 sec for 20 min. The current was applied to the solution in the pipette by means of a silver wire, and the circuit was completed by attaching an alligator clip to the wound margin. Following iontophoresis, the pipette was retracted from the brain, the wound margin closed with clips, and the animal placed on a heating pad until it recovered. The rats were sacrificed 7-14 days post-operatively.

### Perfusion

The rats were administered an overdose of sodium pentobarbital (90 mg/kg, i.p.) and perfused transcardially with 200 ml of phosphate

buffered saline (0.1 M, pH 7.4, 700 ml/h at 4°C) followed by 400 ml of 4.0% paraformaldehyde in 0.1 M phosphate buffer (600 ml/h at 4°C). Immediately prior to infusing the first solution, a bolus of heparin sulfate (300 U in 300 ul) was administered through the aortic cannula. Prior to the infusion of the fixative, the rat was placed on ice. The brain was removed, blocked and post-fixed for 60 min at room temperature. In one rat (FLG-I<sub>3</sub>), the lower medulla and spinal cord also were removed. The brain then was transferred to a solution containing 20% sucrose and 0.01% bacitracin in 0.1 M phosphate buffer, and stored for 1-7 days at 4°C.

#### Tissue Sectioning and Processing

The brains were frozen onto cryostat chucks with O.T.C embedding medium and sectioned in a microtome-cryostat (Hacker-Bright Model 5031, Fairfield, NJ) maintained at -20°C. Coronal sections (18-25 µm) were obtained and placed in ice-cold PBS. Two series of adjacent sections were saved every 100 µm, and float-mounted onto gelatin-chromium potassium sulfate-coated ("subbed") slides. One series was processed for the visualization of Fluoro-Gold labeling and counterstained with 0.01% ethidium bromide (Schmued et al., 1982), while the other was stained with cresyl violet acetate.

#### Immunocytochemistry

##### Colchicine Pretreatment

In order to visualize neurokinin perikarya, colchicine pretreatment was used (Dahlstrom, 1971; Cuello et al., 1983; Skirboll and Hok-

felt, 1983). Bilateral intracerebroventricular (i.c.v.) injections of colchicine (60  $\mu\text{g}$  in 10  $\mu\text{l}$  0.9% saline) were made stereotaxically, under pentobarbital anesthesia (60 mg/kg, i.p.). The coordinates were: anteroposterior,  $\beta + 1.0$  mm; mediolateral,  $\beta \pm 1.0$  mm; dorsoventral,  $\beta - 4.3$  mm. In one rat, an intrathecal injection of colchicine (100  $\mu\text{g}/10$   $\mu\text{l}$ ) was made via PE-10 tubing introduced through the dura at the level of the first cervical vertebra, and gently lowered to the thoracic level. Colchicine infusions were performed 24-48 h prior to sacrifice.

#### Perfusion

The rats were administered an overdose of pentobarbital and perfused transaortically with 200 ml of calcium-free Tyrodes solution (700 ml/h at 4°C) followed by 400 ml of 4.0% paraformaldehyde in 0.1 M phosphate buffer, containing 0.4% saturated picric acid (600 ml/h at 4°C).

#### Antisera

The following primary antisera were used: rabbit anti-SP (Inctar, Inc., Stillwater, MN); rabbit anti-SP (SP2); and, rabbit anti-NKA (NKA2). The SP2 and NKA2 antisera were generously provided by Dr. Ernst Brodin of the Karolinska Institute (Sweden), and have been characterized with respect to their specificity and cross-reactivity (Brodin et al., 1986). All lyophilized antisera were rehydrated in double-distilled water, aliquoted, and stored at -80°C. Thawed antisera were diluted in PBS which contained 0.2-0.3% Triton-X-100 (a detergent which

enhances antibody penetration into tissue) and 0.01% bacitracin (as a bacteriostat). This solution served as the diluent for the secondary antisera as well. The concentrations of primary antisera which provided the best staining were: SP-Incstar, 1:500; SP2 and NKA2, 1:100.

Two different secondary antisera were used. One was a swine anti-rabbit immunoglobulin G (IgG) conjugated to fluorescein-isothiocyanate (FITC; Dakopatts, Sweden) diluted 1:10. The other was a biotinylated goat anti-rabbit IgG (Vector Labs, Burlingame, CA) diluted 1:200. This latter antibody required the use of an avidin-FITC conjugate diluted 1:200 in 10 mM HEPES buffer in 0.9% saline (pH 8.6).

#### Tissue Sectioning and Processing

The tissue was placed in the cryostat at  $-20^{\circ}\text{C}$ , and sectioned as described above. Coronal sections ( $20\ \mu\text{m}$ ) were placed into culture dishes (3-4 per well) and processed free-floating. The  $10\ \mu\text{m}$  sections were thaw-mounted onto "subbed" slides prior to processing. The thaw-mounted sections were brought to room temperature and rehydrated in PBS (10 min). All sections first were incubated in the antibody diluent (PBS with 0.2-0.3% Triton X-100 and 0.01% bacitracin) for 20-30 min. When the avidin-biotin method was used, the diluent also included 5-10% normal goat serum in order to block non-specific avidin binding. Following 3 x 10 min washes in PBS, the sections were incubated in the primary antisera at the appropriate dilution for 24-48 h at  $4^{\circ}\text{C}$ . The sections then were washed in PBS (3 x 10 min) and incubated with the secondary antibody at  $37^{\circ}\text{C}$  (FITC labeled swine anti-rabbit IgG) or room temperature (biotinylated goat anti-rabbit IgG) for 60 min. Following

3 x 10 min washes in PBS, the sections were mounted onto "subbed" slides, air dried, and coverslipped with DePex mounting media which contained 2.0%  $\beta$ -mercaptoethanol to retard fluorescent fading (Dr. C. Saper, University of Chicago, personal communication). Prior to the final washes, the sections which had been processed with the biotinylated secondary antibody were incubated with the FITC labeled-avidin for 60 min at room temperature.

### Microscopy

A Leitz Dialux 20 microscope equipped with a 35 mm camera was used to visualize the FG, FITC, and ethidium bromide staining. Photomicrographs were obtained using epi-illumination and Ploemoptics with the appropriate filter combination. FG labeled cells were examined using a "blue" filter combination (Leitz cube A; 340-380 nm excitation and 430 nm barrier filters). FITC positive material was observed using a "green" filter combination (Leitz cube I<sub>2</sub>; 450-490 nm excitation and 515 nm barrier filters). Ethidium bromide staining was visualized using a "red" filter combination (Leitz N<sub>2</sub> cube; 530-560 nm excitation and 580 nm barrier filters). Photomicrographs were obtained with either black and white print (T-Max; ASA 400) or color slide (Ektachrome; ASA 400; Eastman Kodak Co., Rochester, NY) film.

## CHAPTER IV

### RESULTS

#### A. Behavioral Studies

##### Experiment I: DiMe-C7 Dose-Response

###### Procedure

In the first experiment we replicated and extended our earlier findings that intra-MR DiMe-C7 dose-dependently increases LMA (Paris and Lorens, 1987 a,b), using a wider range of doses. Following five days of habituation to the apparatus, the rats (n = 10) received seven intra-MR injections, each separated by 48 h. Vehicle (1.0  $\mu$ l) and six doses (11.4, 34.2, 114, 342, 1140 and 3420 pmol) of DiMe-C7 were administered once according to a randomized design.

###### Results

The cannulae placements in the 10 rats used in this study are shown schematically in Appendix II (Fig A). As we have discussed previously (Paris and Lorens, 1987 a), the repeated drug administration protocol used in the present studies (one injection every other day) not only yields very reproducible behavioral effects with no apparent tolerance development, but minimal glial scarring (Fig. 1).

In agreement with our previous reports (Paris and Lorens, 1987 a,b), intra-MR infusions of DiMe-C7 produce dose-dependent increases in

LMA (Fig. 2). An ANOVA revealed a significant drug treatment effect [ $F(6,54) = 28.8, p < 0.001$ ]. A post-hoc comparison of means indicated that LMA was significantly ( $p < 0.01$ ) elevated following all doses of DiMe-C7 except the 11.4 pmol dose. The effects of the two highest doses, 1140 and 3420 pmol, were not significantly different from each other, but were significantly greater than those of the lower doses. In addition, the effects of the 342 pmol dose were significantly greater than those after the 34.2 pmol dose.

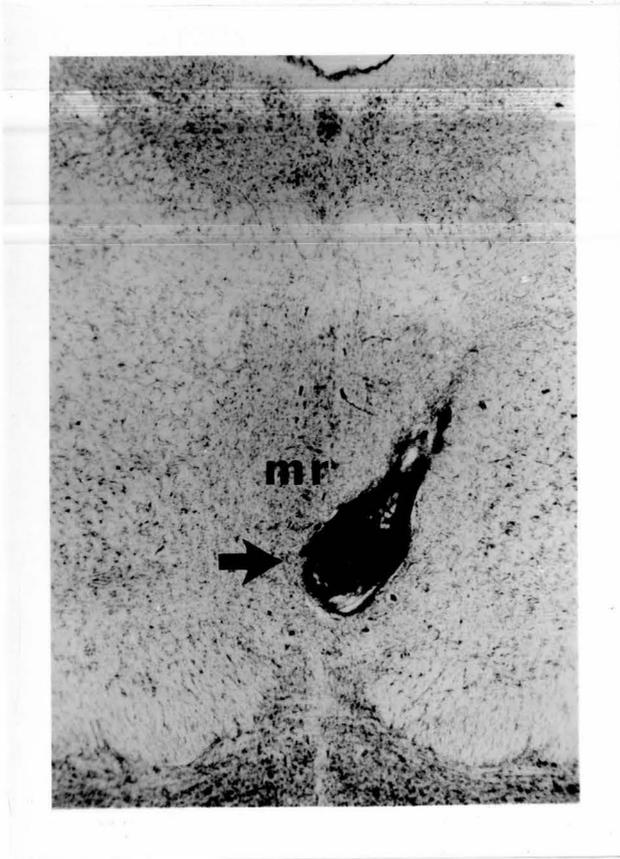


Fig. 1. MR Cannula Placement: Photomicrograph (x25) of a 50  $\mu\text{m}$  coronal section (cresyl violet stain) showing a cannula tip (arrow) which terminated in the ventral part of the median raphe nucleus (MR) in an animal from Experiment II. The cannula tracks in all rats were associated with glial scarring which approximated 0.5 mm in diameter.

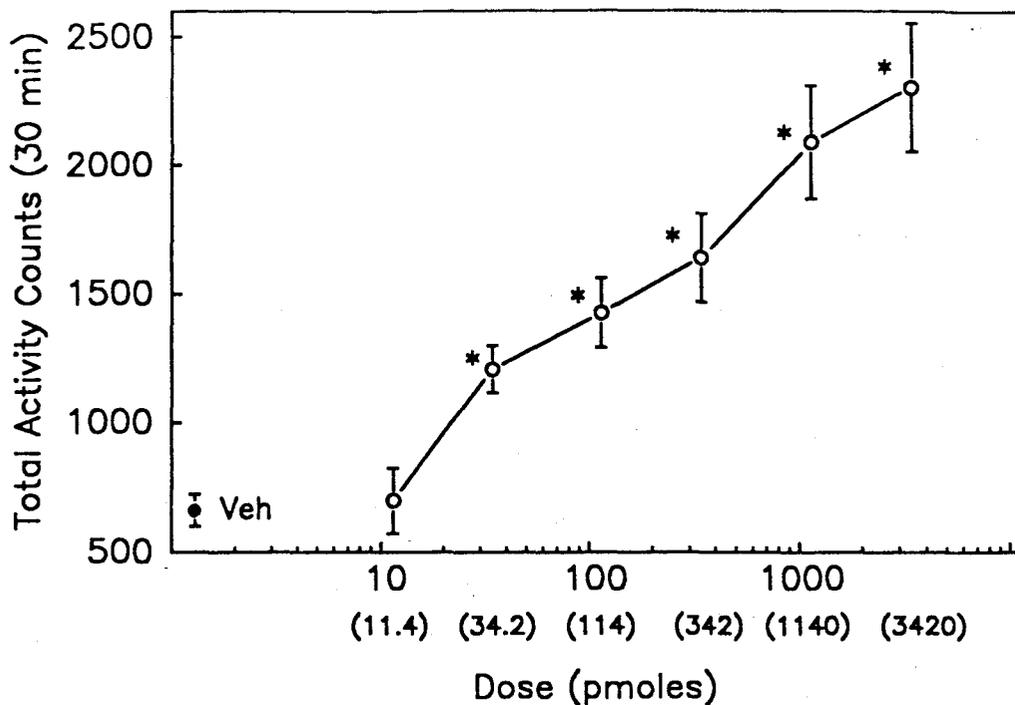


Fig. 2. DiMe-C7 Dose-Response: Total activity for 30 min post-injection of vehicle (Veh) and different doses of DiMe-C7. Data represent mean  $\pm$  SEM for  $n = 10$  rats. Significant difference from corresponding Veh: \*  $p < 0.05$  (ANOVA followed by Duncan's multiple range test). X-axis is a log scale.

## Experiment II: Non-Mammalian Tachykinin Dose-Responses

### Procedure

In this experiment, the non-mammalian tachykinins, ELE, KAS and PHY, were tested. Three separate groups of rats ( $n = 7 - 10$  per group) were used, one for each peptide tested.

A linear regression analysis of the results from the first experiment indicated that three doses of DiMe-C7 produced increases in activity which were approximately 30, 50 and 90% of the maximal response. These doses were: 30, 230 and 1140 pmol, respectively. The same three doses of the tachykinins were selected in order to compare their effects on LMA. In addition, all rats received an injection of a sub-maximal dose of DiMe-C7 (230 pmol) for purpose of comparison.

Following the five day habituation period, the rats received five intra-MR injections, each separated by 48 h. Vehicle ( $1.0 \mu\text{l}$ ), a test dose of DiMe-C7 (230 pmol), and the three doses of the particular peptide (30, 230 and 1140 pmol) were administered according to a randomized design.

### Results

The cannulae placements for the 24 rats used in this study are depicted schematically in Appendix II (Fig. A).

Fig. 3 shows the effects of the non-mammalian tachykinins on LMA. An initial analysis confirmed that there were no significant group differences in the activity levels following the injections of vehicle [ $F(2,21) = 2.23, p > 0.10$ ] or DiMe-C7 [ $F(2,21) = 2.95, p > 0.10$ ].

Therefore, for the sake of simplicity, the vehicle and DiMe-C7 scores were combined for all three groups for both statistical and graphical purposes.

Further analysis of the 30 min post-drug activity scores indicated a significant group effect [ $F(2,21) = 23.48, p < 0.001$ ] and a significant effect of peptide dose [ $F(4,84) = 24.60, p < 0.001$ ]. The interaction between the group and dose factors also was significant [ $F(8,84) = 2.19, p = 0.05$ ].

All doses of ELE significantly elevated LMA. The activity levels observed following infusions of the 30 and 230 pmol doses were not significantly different from each other, but were significantly less than after infusions of the 1140 pmol dose. The effect produced by 1140 pmol of ELE was equivalent to that produced by the 230 pmol test dose of DiMe-C7.

A post-hoc comparison revealed that only the two highest doses of KAS tested, 230 and 1140 pmol, significantly elevated LMA compared to vehicle. In addition, the activity levels following all doses tested were significantly less than those induced by the DiMe-C7 test dose (230 pmol). Although only the two highest doses tested produced LMA effects which were significantly different from those following the vehicle injections ( $p < 0.05$ ), the LMA effects after these three doses of KAS did not significantly differ from each other.

For PHY, only the two highest doses, 230 and 1140 pmol, significantly elevated LMA in comparison to vehicle injections. The LMA following infusions of all doses tested was significantly less ( $p < 0.05$ )

than that produced by DiMe-C7 (230 pmol). The effects of the three PHY doses, furthermore, did not significantly differ from each other.

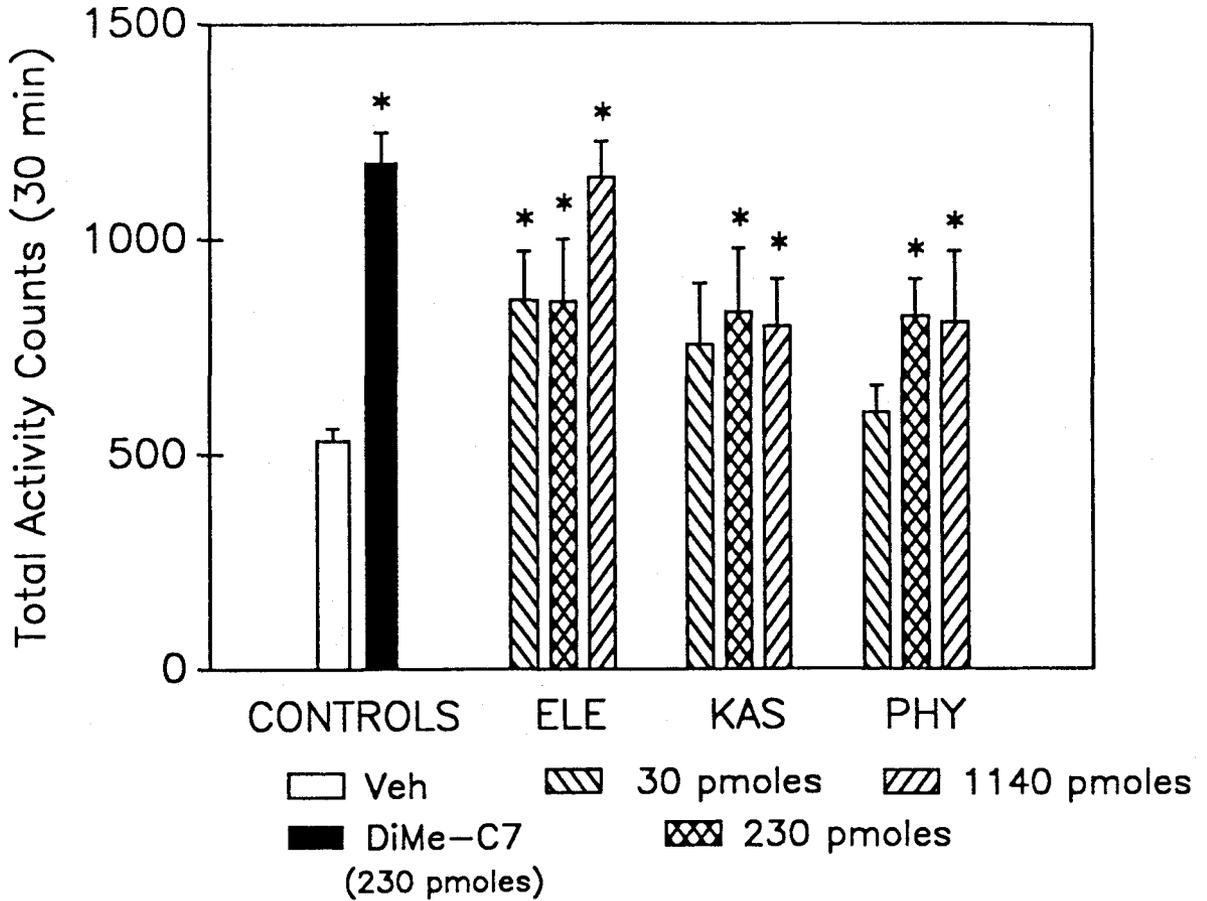


Fig. 3. Tachykinin Dose-Response: Total activity for 30 min post-injection of vehicle (Veh), DiMe-C7 and three doses of eleo-  
doisin (ELE), kassinin (KAS) and physalaemin (PHY) in separate  
groups of rats (n = 7-9 per group). Data represent mean  $\pm$  SEM.  
Significant difference from Veh: \* p < 0.05 (ANOVA followed by  
Duncan's multiple range test).

## Experiment III: Mammalian Neurokinin Dose-Responses

### Procedure

This experiment was conducted identically to Experiment II except for the peptides tested. The mammalian tachykinins, neurokinin A and substance P, and the neurokinin B agonist, senktide, were tested.

### Results

The cannula placements for the 21 rats used in this study are depicted schematically in Appendix II (Fig A).

An initial analysis of the 30 min post-drug total activity scores following vehicle [ $F(2,18) = 1.02, p > 0.3$ ] and DiMe-C7 [ $F(2,18) = 0.46, p > 0.3$ ] injections confirmed that there were no significant differences between the three groups of rats. Therefore, both the vehicle and DiMe-C7 scores for all three groups were combined for statistical and graphical purposes.

Fig. 4 shows that all three of the neurokinins increased LMA. An ANOVA indicated that there was a significant effect of the dose of the particular peptide [ $F(4,72) = 26.14, p < 0.001$ ], as well as a significant difference between the three groups [ $F(2,18) = 9.60, p < 0.001$ ]. The interaction between dose and group also was significant [ $F(8,72) = 8.59, p < 0.01$ ].

For the senktide group, all three doses (30, 230, and 1140 pmol), significantly increased LMA compared to the effects of both vehicle and DiMe-C7. Further analysis indicated that the LMA increases produced by the 230 pmol dose was significantly greater than that produced by 30

pmol dose, and that the hyperactivity produced by the 1140 pmol dose was significantly greater than that following the 30 and 230 pmol doses.

All three doses of NKA significantly elevated LMA compared to vehicle. The hyperactivity produced by 1140 pmol was significantly greater than that induced by the 30 and 230 pmol doses, as well as the DiMe-C7 test-dose (230 pmol). The increases in LMA produced by the 30 and 230 pmol doses of NKA did not differ significantly from each other, or from those observed following DiMe-C7 (230 pmol) administration.

For the SP group, only the highest dose tested (1140 pmol) significantly increased LMA compared to vehicle. The increase in LMA produced by the 1140 pmol dose was significantly greater than that observed following the 30 pmol SP dose, but did not differ from the increase in activity produced by the DiMe-C7 test-dose.

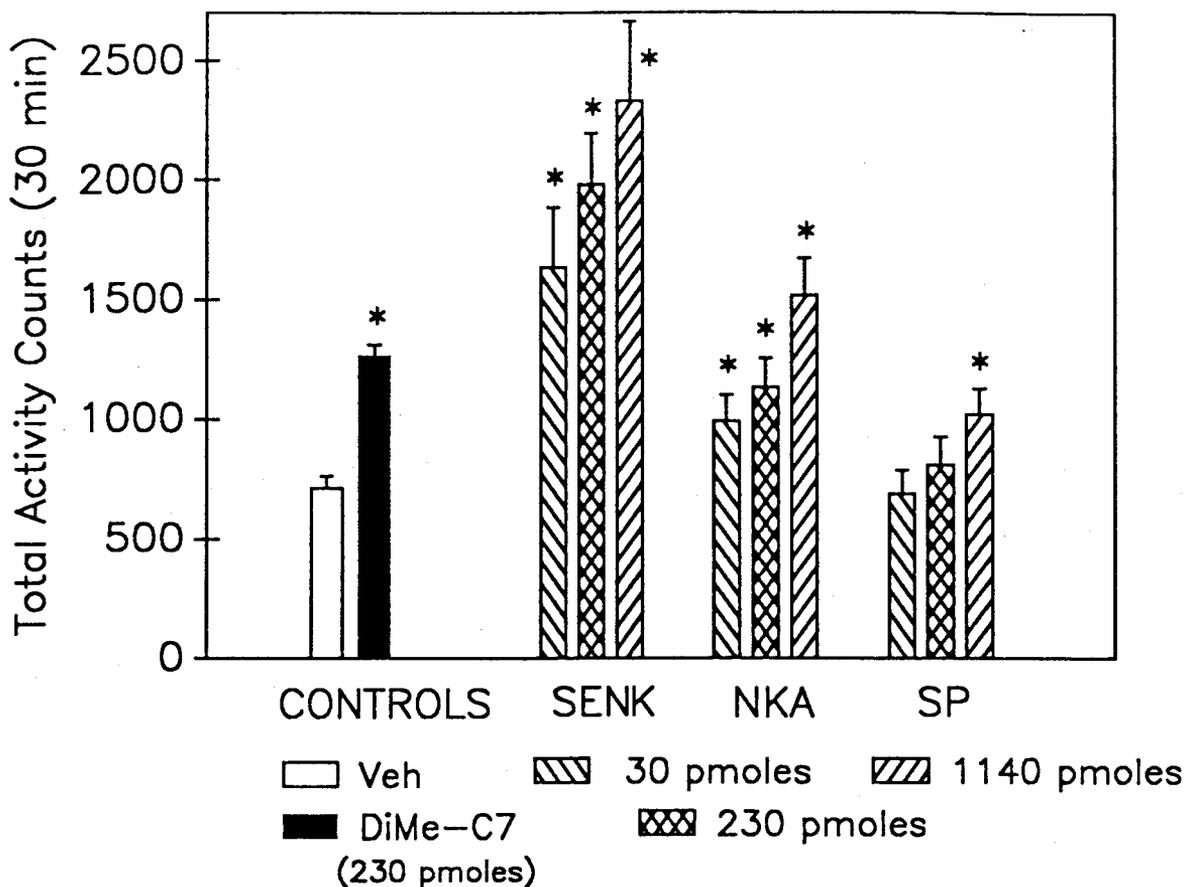


Fig. 4. Neurokinin Dose-Response: Total activity for 30 min post-injection of vehicle (Veh), DiMe-C7 and three doses of senktide (SENK), neurokinin A (NKA) and substance P (SP) in separate groups of rats ( $n = 8$  per group). Data represent mean  $\pm$  SEM. Significant difference from Veh: \*  $p < 0.05$  (ANOVA followed by Duncan's multiple range test).

## Experiment IV: NKA Low Dose-Response Study

### Procedure

Following the five day habituation period, the rats ( $n = 7$  per experiment) received five intra-MR injections each separated by two days. Vehicle ( $1.0 \mu\text{l}$ ) and four doses (1.0, 3.0, 10 and 30 pmol) of NKA were administered according to a randomized design. Prior to sacrifice and histological analysis, some of the rats were used in Experiment VI.

### Results

The cannulae placements for the eight rats used in this study are shown schematically in Appendix II (Fig. A).

Fig. 5 shows that NKA dose-dependently increased LMA. An ANOVA indicated that there was a significant effect of NKA on LMA [ $F(4,28) = 13.8$ ,  $p < 0.001$ ]. A post-hoc comparison of means revealed that only the 30 pmol dose significantly elevated LMA compared to the vehicle injections. The increase in LMA produced by the 30 pmol NKA dose was equivalent to that observed in Experiment III (Fig.4). As seen in Fig. 5, injections of 1.0 and 3.0 pmol of NKA decreased LMA, however, this was not statistically significant ( $0.05 < p < 0.10$ ).

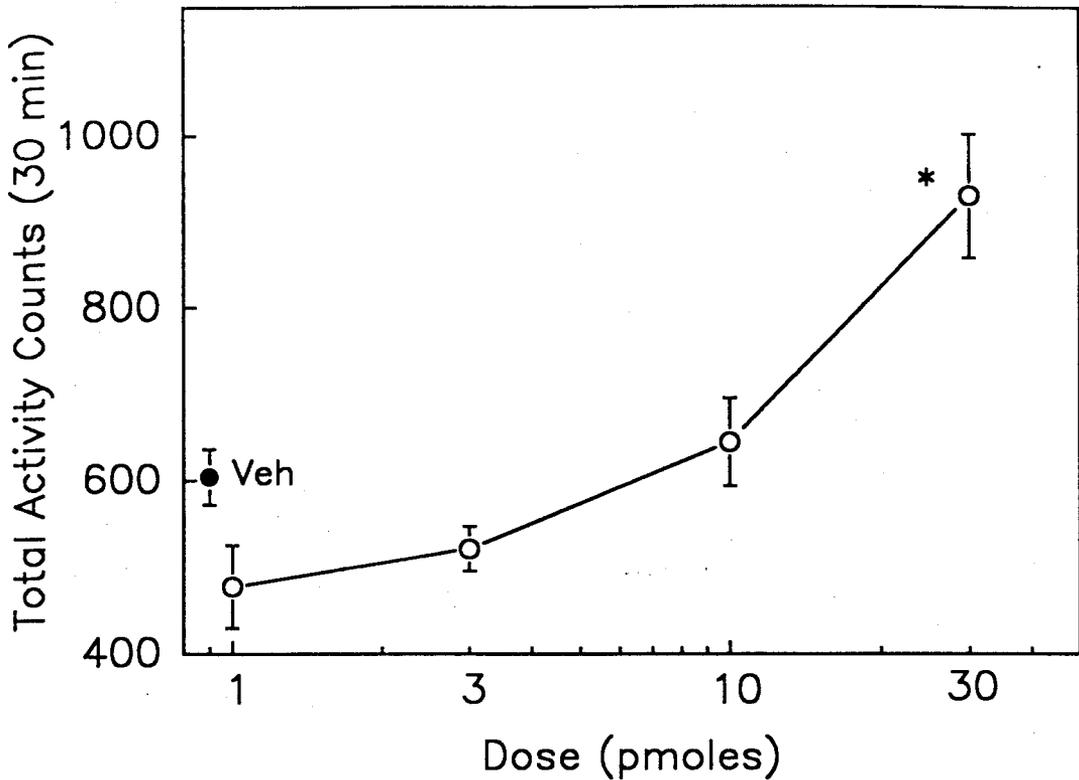


Fig. 5. Neurokinin A Low Dose-Response: Total activity for 30 min post-injection of vehicle (Veh) and varying doses of neurokinin A. Data represent mean  $\pm$  SEM for  $n = 8$  rats. Significant difference from corresponding Veh: \*  $p < 0.05$  (ANOVA followed by Duncan's multiple range test). X-axis is a log scale.

## Experiment V: Senktide Low Dose-Response Study

### Procedure

This experiment was conducted identically to Experiment IV except for the peptide studied. Senktide was tested at the same four doses used in the previous experiment.

### Results

The cannulae placements for the 8 rats used in this study are schematically represented in Appendix II (Fig. A).

Fig. 6 shows that senktide dose-dependently increased LMA. An ANOVA indicated that there was a significant effect of senktide on LMA [ $F(4,28) = 16.5, p < 0.001$ ]. Post-hoc comparisons of means showed that all of the senktide doses tested, except 1.0 pmol, significantly elevated LMA. The increase in LMA produced by the 30 pmol dose was equivalent to that observed in Experiment III (Fig. 4). Further analysis showed that the increasing doses of senktide each produced significantly greater effects on LMA than the immediately lower dose. Thus, the LMA effect of the 30 pmol dose was significantly greater ( $p < 0.05$ ) than that following 10 pmol, which was greater than that following 3.0 pmol, which was greater than that following the 1.0 pmol dose, which was equal to that following vehicle injections.

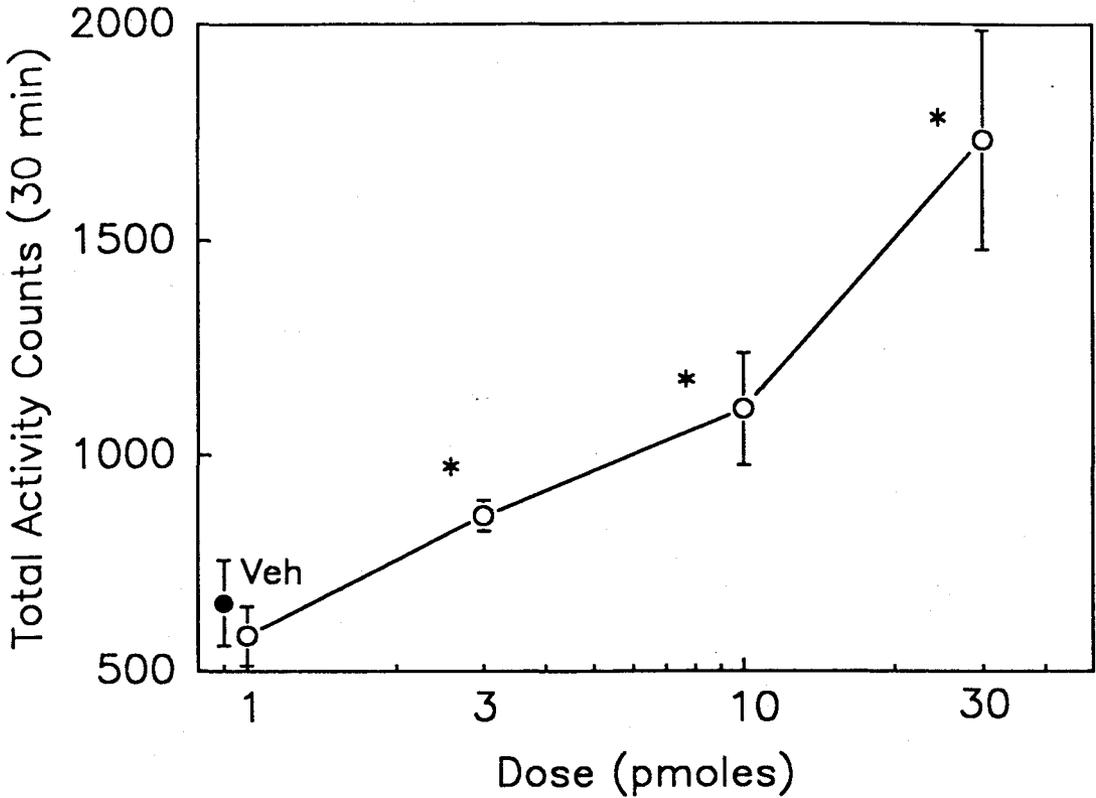


Fig. 6. Senktide Low Dose-Response: Total activity for 30 min post-injection of vehicle (Veh) and varying doses of senktide. Data represent mean  $\pm$  SEM for  $n = 8$  rats. Significant difference from corresponding Veh: \*  $p < 0.05$  (ANOVA followed by Duncan's multiple range test). X-axis is a log scale.

### Regression Analysis

Fig. 7 shows the regression analysis performed for the tachykinins and analogues studied. An initial analysis of activity following vehicle infusions showed that there were no significant differences between the groups of rats [ $F(8,62) = 2.02$ ;  $p > 0.05$ ]. The grand mean  $\pm$  SEM for all the vehicle scores was  $624 \pm 24$ . Analysis of the DiMe-C7 test scores also did not reveal any significant group differences [ $F(5,39) = 1.95$ ;  $p > 0.10$ ]. The grand mean  $\pm$  SEM for all DiMe-C7 test scores was  $1220 \pm 43$ .

Tests for parallelism indicated that the senktide and DiMe-C7 regression lines were not significantly different ( $t = 0.56$ ,  $df = 8$ ;  $p > 0.05$ ).<sup>\*</sup> Both the senktide and DiMe-C7 slopes, however, were significantly different from the SP and NKA lines. The SP and NKA regression lines, moreover, did not differ significantly ( $t = 1.54$ ,  $df = 2$ ;  $p > 0.05$ ). The relative positions of the lines indicate that senktide was more potent than DiMe-C7, and NKA was more potent than SP.

Analysis of the regression lines for the non-mammalian tachykinins indicated that they were parallel, and that their slopes were significantly different from those of senktide and NKA, but not SP. It should be noted, however, that the slopes for the non-mammalian compounds were relatively flat indicating a lack of dose-dependent effects within the range of doses tested.

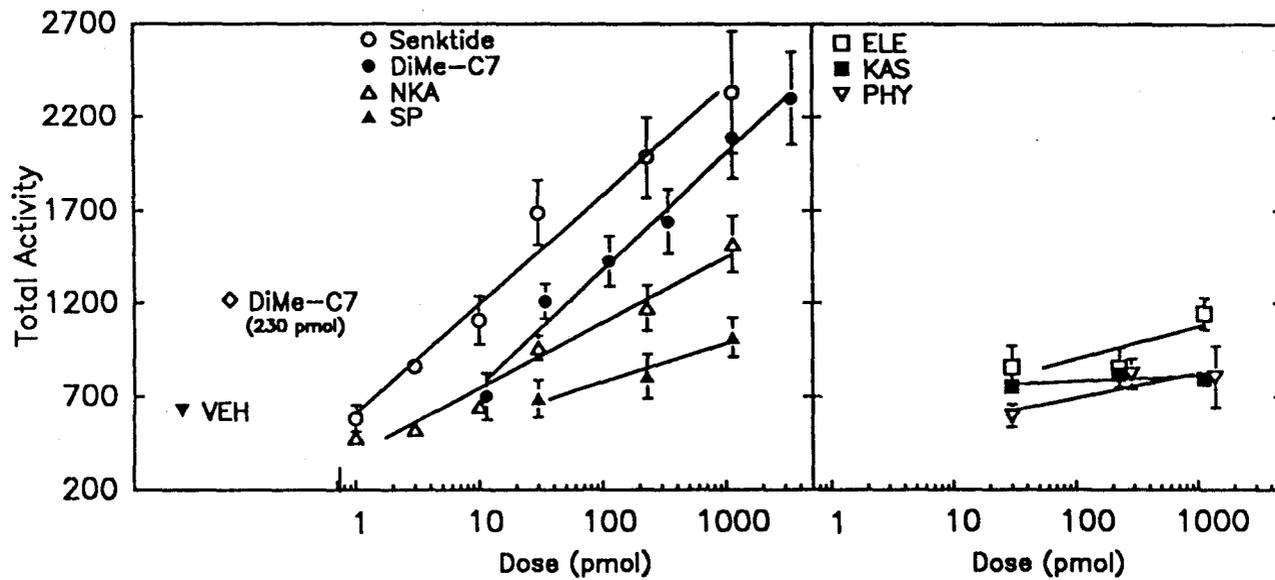
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<sup>\*</sup> For the sake of clarity, only the most relevant t-values are given.

Fig. 7. Regression Analysis: Regression lines of total activity for 30 min post-injection versus tachykinins dose in pmol (log scale).

Regression lines:   senktide:  $y = 585(x) + 608$   
                      DiMe-C7:  $y = 622(x) + 138$   
                      NKA:      $y = 345(x) + 393$   
                      SP:       $y = 128(x) + 578$   
                      ELE:      $y = 87(x) + 781$   
                      KAS:      $y = 22(x) + 751$   
                      PHY:      $y = 89(x) + 566$

Veh = grand mean of vehicle activity scores; DiMe-C7 (230 pmol)  
= grand mean of test dose activity scores.



## Experiment VI: Intra-MR NKB Injections

### Procedure

Rats from Experiments IV ( $n = 4$ ) and V ( $n = 5$ ), as well as two naive rats, were habituated to the testing apparatus for five days. On the sixth day the rats received an injection of either the formic acid vehicle ( $1.0 \mu\text{l}$ ) or NKB ( $3.0 \text{ pmol}$ ). They were sacrificed by decapitation immediately following removal from the testing chamber.

### Results

The cannulae placements in the rats which received injections of either the formic acid vehicle or NKB are included in Appendix II (Fig. A). The cannulae placements for the two additional rats (not shown) were comparable.

Fig. 8 shows the 30 min total activity following either formic acid vehicle or NKB ( $3.0 \text{ pmol}$ ) injections. NKB significantly elevated LMA ( $t = 3.31$ ,  $df = 9$ ,  $p < 0.01$ ). For the purpose of comparison, the activity levels obtained in Experiment V following injections of vehicle or senktide ( $3.0 \text{ pmol}$ ) are presented in Fig. 8. NKB elevated LMA to a level equivalent to that seen with an equimolar dose of senktide ( $t = 1.94$ ,  $df = 11$ ,  $p > 0.05$ ).

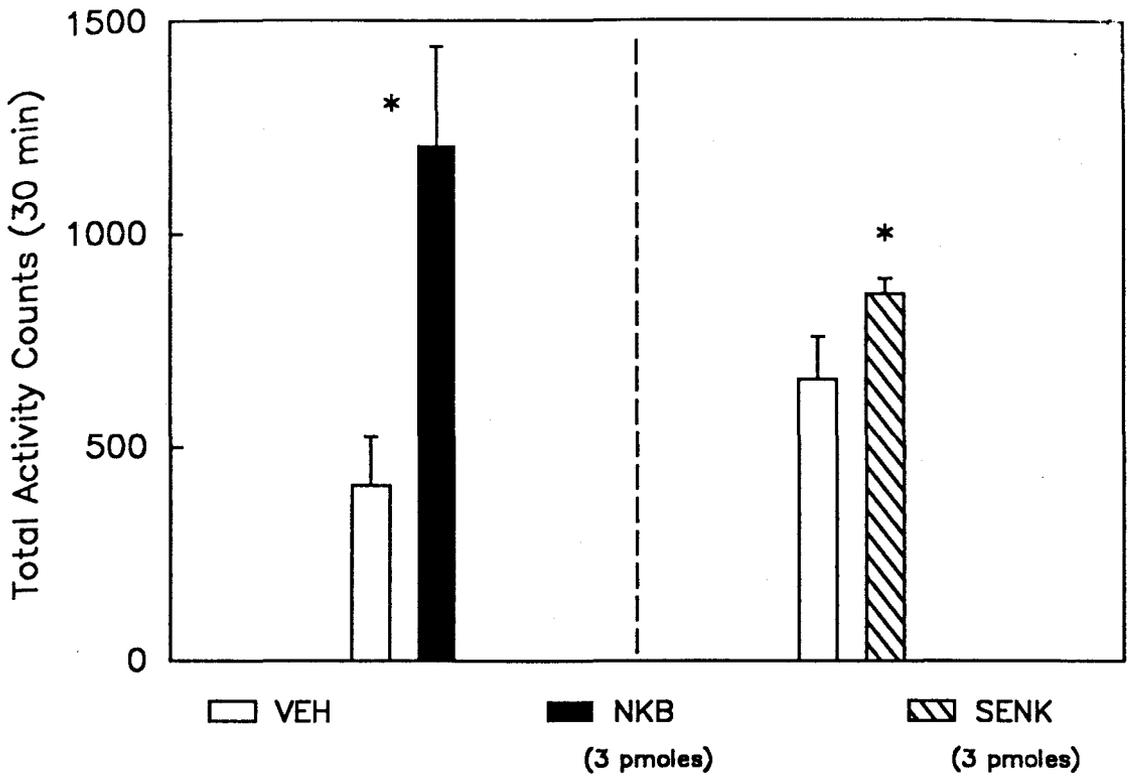


Fig. 8. Neurokinin B Infusions: Total activity for 30 min post-injection of vehicle (Veh), neurokinin B (NKB) and senktide (SENK; from Experiment V). Data represent mean  $\pm$  SEM. Significant difference from corresponding Veh: \*  $p < 0.05$  (Student's t-test).

## Experiment VII: 5,7-Dihydroxytryptamine (5,7-DHT) Lesions

### Procedure

Beginning 14 days post-operatively, and following two days of handling, the animals were habituated to the testing apparatus for five days, 60 min per day. The day immediately following the last day of habituation, drug testing was begun. At 48 h intervals, each rat (n = 32) received intra-MR injections of vehicle (1.0  $\mu$ l), DiMe-C7 (1140 pmol), NKA (230 pmol) and senktide (30 pmol). These doses were selected on the basis of our previous dose-response analysis which showed that they produced sub-maximal but equivalent increases in LMA (see Fig. 2 and 3). Each drug was administered only once, according to a randomized design.

### Results

The cannulae placements in the 21 rats used in this study are shown schematically in Appendix II (Fig. B).

The 5,7-DHT injections into the MR produced some cell loss (presumably 5-HT neurons) with minimal glial scarring in the region surrounding the cannula tip. No extensive scarring or cavitation was observed. Analysis of the MFB injection sites revealed that the 5,7-DHT and vehicle infusions did not cause any destruction of perikarya in the region, or any cavitation at the tip of the injection needle. No evidence of retrograde degeneration in the MR was obtained.

An initial analysis of the data (not shown) indicated that the two groups of control animals did not differ either in their response

to the neurokinin infusions or neurochemically, therefore their data were combined for subsequent analysis.

Fig. 9 shows the total activity counts for the 30 min following the injection of DiMe-C7, NKA and senktide for the three groups of rats. An ANOVA revealed significant lesion [ $F(2,24) = 6.18; p < 0.01$ ] and drug [ $F(3,72) = 24.91; p < 0.001$ ] effects. The lesion x drug interaction also was significant [ $F(6,72) = 4.63; p < 0.001$ ]. A post-hoc analysis indicated that for the control group, administration of all three peptides significantly elevated LMA compared to vehicle, but that the activity levels produced by these infusions did not significantly differ from each other. Further analysis indicated that the LMA following vehicle infusions was equivalent for all three groups. The activity levels produced by infusions of senktide and DiMe-C7 were equivalent between the control and MFB lesion groups.

The MR 5,7-DHT lesions completely blocked the LMA produced by infusions of DiMe-C7, NKA and senktide. In contrast, the MFB 5,7-DHT lesions did not affect the locomotor hyperactivity produced by intra-MR administration of DiMe-C7 and senktide, but appeared to attenuate the effects of NKA. The hyperactivity produced by infusions of NKA was significantly lower than that produced by both senktide and DiMe-C7 in rats with MFB lesions. In addition, the hyperactivity produced by infusions of NKA in the MFB lesion group was intermediate to that produced by similar infusions in the control and MR lesion groups.

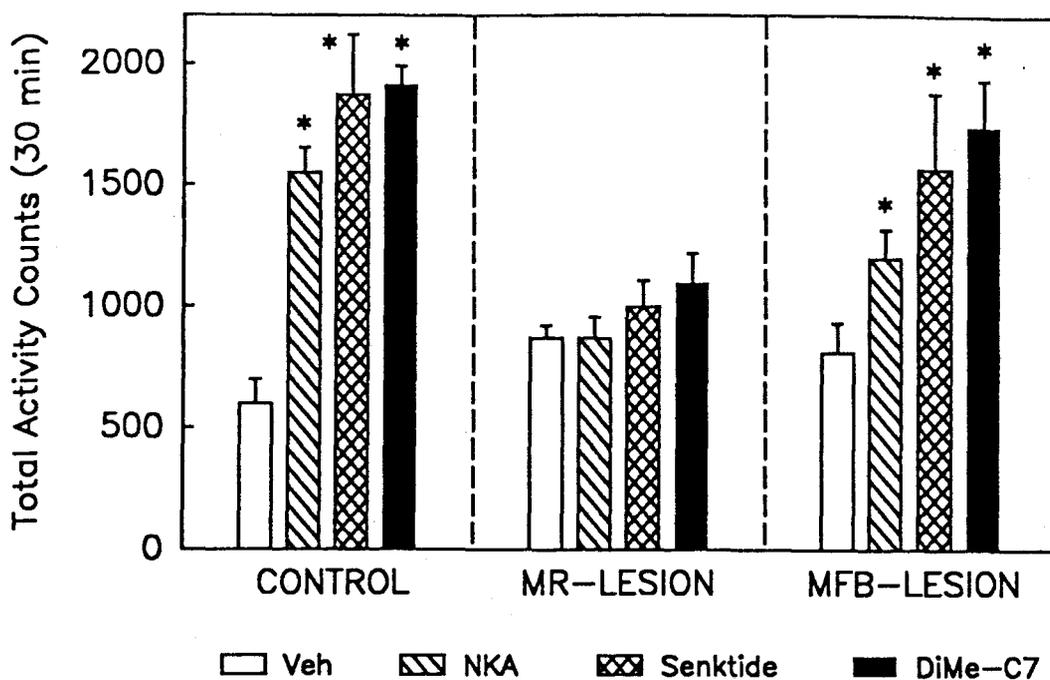


Fig. 9. MR/MFB 5,7-DHT Lesions: Effects of intra-median raphe (MR) infusions of senktide (30 pmoles), neurokinin A (NKA; 230 pmoles), DiMe-C7 (1140 pmoles) and vehicle (1.0  $\mu$ l PBS with acetic acid, pH 7.2) in rats which had sustained either MR or MFB 5,7-dihydroxytryptamine lesions, or vehicle control injections, two weeks previously (n = 9 per group). Data represent mean  $\pm$  SEM total activity counts for 30 min post-injection. Significant difference from corresponding vehicle infusion: \* =  $p < 0.05$ .

The MR 5,7-DHT injections significantly reduced the 5-HT and 5-HIAA concentrations in all five brain regions assayed (Table II). The MFB 5,7-DHT injections produced virtual depletion of 5-HT and 5-HIAA concentrations in all the brain regions. In all cases, the MFB lesions produced reductions greater than 86% from control levels.

Table III shows the effects of the MR and MFB 5,7-DHT lesions on forebrain DA, NE and metabolite levels. The MFB lesions produced significant reductions in NE levels in both the MFC and hippocampus. These lesions also produced significant reductions in DA levels in all brain regions analyzed, except the amygdala. DOPAC levels were affected by the MFB lesions only in the striatum and nucleus accumbens. HVA concentrations were significantly reduced only in the nucleus accumbens of rats with MFB lesions.

The only effect of the MR lesions on DA levels was an increase in the amygdala. Similarly, DOPAC levels were significantly increased in both the amygdala and MFC in rats with MR 5,7-DHT lesions.

Table II:

EFFECTS OF INTRA-MEDIAN RAPHE (MR) AND MEDIAL FOREBRAIN BUNDLE (MFB)  
5,7-DIHYDROXYTRYPTAMINE LESIONS ON FOREBRAIN  
SEROTONIN LEVELS

<u>Region</u> <u>Group</u>	<u>5-HT</u> (ng/g)		<u>5-HIAA</u> (ng/g)
<u>Amygdala</u>			
Control	1114 ± 58		255 ± 17
MR-Lesion	* 292 ± 74 (-74%)	+	89 ± 19 (-65%)
MFB-Lesion	* 60 ± 29 (-95%)		N.D. (- >96%)
-----			
<u>Medial Frontal Cortex</u>			
Control	792 ± 44		192 ± 11
MR-Lesion	* 222 ± 71 (-72%)	*	49 ± 17 (-74%)
MFB-Lesion	* 42 ± 16 (-95%)		N.D. (- >97%)
-----			
<u>Striatum</u>			
Control	794 ± 47		319 ± 23
MR-Lesion	* 429 ± 68 (-46%)	*	158 ± 32 (-50%)
MFB-Lesion	* 114 ± 8 (-86%)		N.D. (- >94%)
-----			
<u>Nucleus Accumbens</u>			
Control	1157 ± 83		327 ± 18
MR-Lesion	+ 652 ± 155 (-44%)	*	167 ± 38 (-49%)
MFB-Lesion	N.D. (- >98%)		N.D. (- >94%)
-----			
<u>Hippocampus</u>			
Control	583 ± 37		278 ± 17
MR-Lesion	* 67 ± 13 (-89%)	*	61 ± 9 (-78%)
MFB-Lesion	* 53 ± 16 (-91%)	*	30 ± 9 (-89%)

N = 7-9 per group; data represent mean ± SEM; numbers in parenthesis = % change from corresponding Control; significant difference from corresponding Control: + = p < 0.05; \* = p < 0.01.

N.D. = Not detectable; less than limits of sensitivity: amygdala, < 10 ng/g; medial frontal cortex, < 5 ng/g; striatum, < 20 ng/g; and nucleus accumbens, < 20 ng/g wet weight of tissue.

Table III:

EFFECTS OF INTRA-MEDIAN RAPHE (MR) AND MEDIAL FOREBRAIN BUNDLE (MFB)  
5,7-DIHYDROXYTRYPTAMINE LESIONS ON FOREBRAIN DOPAMINE AND  
NOREPINEPHRINE LEVELS

<u>Region</u> <u>Group</u>	<u>NE</u>	<u>DA</u>	<u>DOPAC</u>	<u>HVA</u>
	(ng/g)			
<u>Amygdala</u>				
Control	597 ± 34	381 ± 42	30 ± 3	---
MR-Lesion	659 ± 36	* 503 ± 21 (+32%)	* 43 ± 4 (+43%)	---
MFB-Lesion	476 ± 22	299 ± 22	29 ± 2	---
-----				
<u>Medial Frontal Cortex</u>				
Control	353 ± 17	105 ± 9	18 ± 1	---
MR-Lesion	351 ± 18	130 ± 11	* 29 ± 3 (+61%)	---
MFB-Lesion	* 268 ± 16 (-24%)	* 83 ± 9 (-21%)	16 ± 2	---
-----				
<u>Striatum</u>				
Control	---	7678 ± 346	887 ± 67	352 ± 26
MR-Lesion	---	7543 ± 358	1003 ± 29	412 ± 26
MFB-Lesion	---	* 5737 ± 406 (-25%)	* 533 ± 60 (-40%)	264 ± 40
-----				

Table III (cont'd)

Nucleus Accumbens

Control	---	7222 ± 332	1109 ± 49	378 ± 27
MR-Lesion	---	6251 ± 371	1088 ± 101	372 ± 45
MFB-Lesion	---	* 5682 ± 141 (-21%)	* 760 ± 78 (-31%)	* 231 ± 31 (-39%)

-----  
Hippocampus

Control	808 ± 31	---	---	---
MR-Lesion	831 ± 53	---	---	---
MFB-Lesion	* 590 ± 45 (-27%)	---	---	---

N = 7-9 per group; data represent mean ± SEM; numbers in parenthesis = % change from corresponding control; significant difference from corresponding control: \* = p < 0.01.

Experiment VIII: DiMe-C7 Dose-Response in a Novel Open FieldProcedure

Rats (n = 30 from Experiment II) were handled daily for 3 days. They then were tested (10 rats per day for three days) following an infusion of either vehicle (1.0  $\mu$ l) or three doses of DiMe-C7 (30, 230, and 1140 pmol). Each animal was injected with drug or vehicle and immediately placed in the center of the arena. The behavior of the animal was videotaped for 30 min for subsequent analysis. Following the testing period, each rat was removed, transported to another room and swiftly decapitated. The brain was removed and placed in buffered formalin for subsequent histological analysis.

Results

The effects of DiMe-C7 on behavior in a novel open field are shown in Fig. 10. An analysis of the total activity (wall squares entered + center squares entered + rears + head pokes) indicated that DiMe-C7 did not affect activity in a novel open field [ $F(3,21) = 0.55$ ;  $p > 0.3$ ]. Further analysis of each component behavior (Fig. 11) demonstrated no significant effects of DiMe-C7: wall squares entered [ $F(3, 20) = 0.07$ ;  $p > 0.3$ ]; center squares entered [ $F(3,20) = 1.32$ ;  $p > 0.05$ ]; rears [ $F(3,20) = 2.78$ ,  $p > 0.05$ ]; and head pokes [ $F(3,20) = 0.74$ ;  $p > 0.3$ ]. There were significant effects of time (Fig. 11) within the chamber for wall squares entered [ $F(9,27) = 3.72$ ;  $p < 0.01$ ], center squares entered [ $F(9,180) = 3.65$ ;  $p < 0.05$ ], and head pokes [ $F(9,180) = 5.53$ ;  $p < 0.01$ ]. The only significant drug x time inter-

actions were for rears [ $F(27,180) = 1.58$ ;  $p = 0.05$ ] and head pokes [ $F(27,180) = 2.08$ ;  $p < 0.01$ ]. Analysis of the time effects indicated that the greatest contribution came from the highest dose of DiMe-C7 (1140 pmol). There was a general increase in activity over the 30 min testing period.

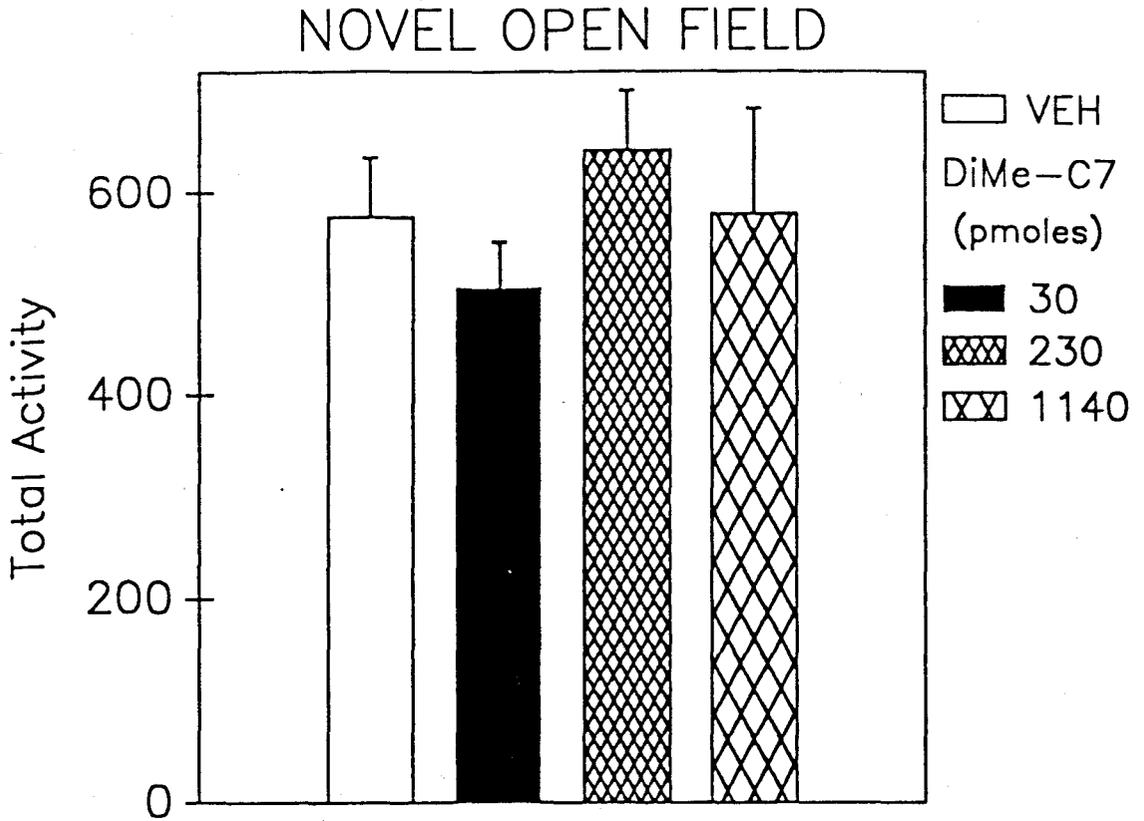


Fig. 10. DiMe-C7 Novel Open Field: Total Activity: Total activity (wall squares + center squares + rears + head pokes) for 30 min following infusion of varying doses of DiMe-C7. N = 7/9 rats per dose; data represent mean  $\pm$  SEM. No significant differences from vehicle infusions were observed.

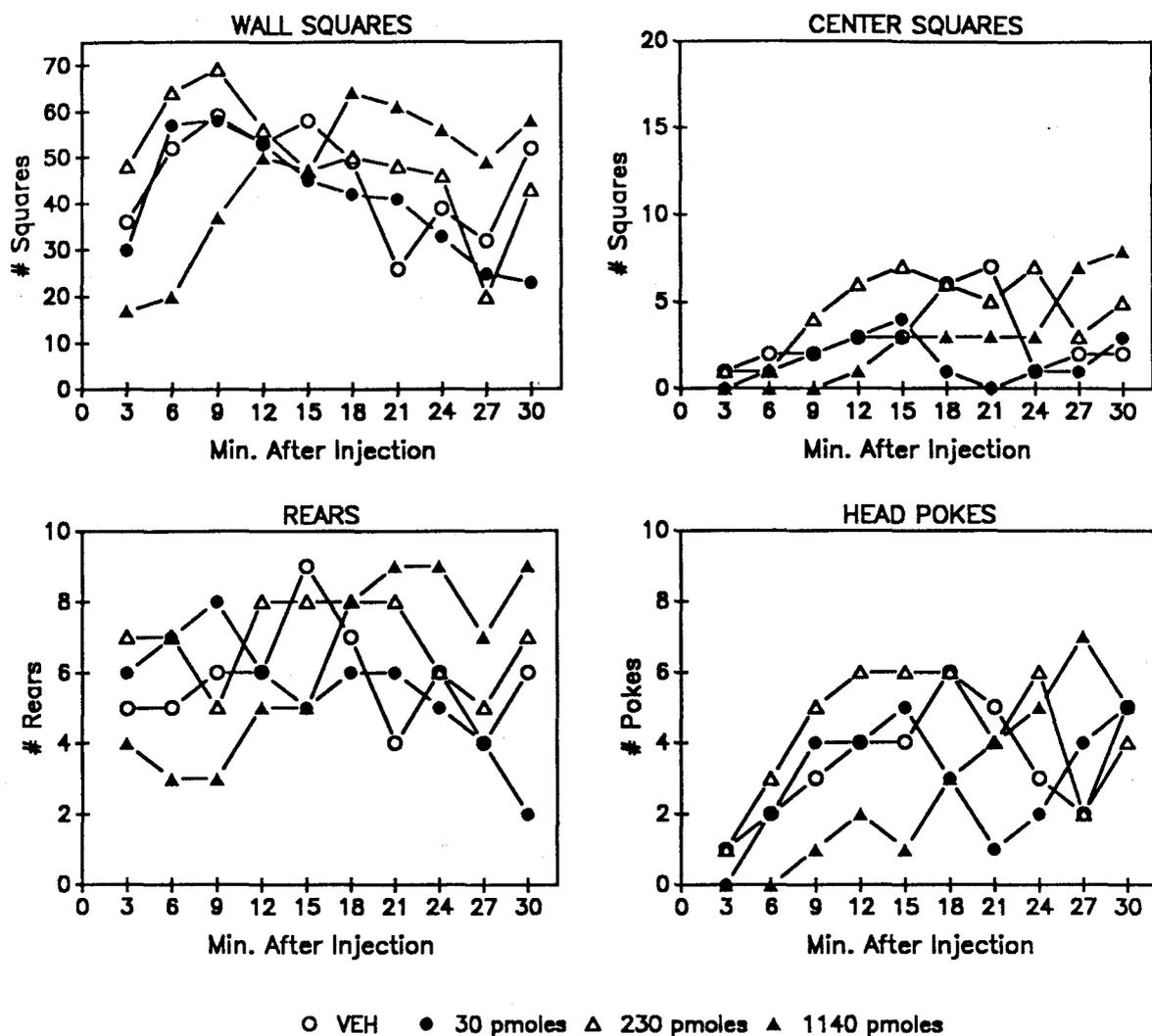


Fig. 11. DiMe-C7 Novel Open Field: Component Behaviors: Number of wall squares, center squares, rears and head pokes per 3 min block for 30 min following infusion of varying doses of DiMe-C7. N = 7/9 rats per dose; data represent group mean; error bars were omitted for clarity. No significant differences from vehicle infusions were observed.

Experiment IX: Neurokinin Effects on Novel Open Field BehaviorProcedure

Rats (n = 30 from Experiment III) were handled daily for three days beginning eight days following the completion of Experiment III. This experiment was conducted identically to Experiment VII, except for the drugs tested. In this study, each rat was infused with either vehicle (1.0  $\mu$ l), NKA (230 pmol) or senktide (30 pmol). Following the completion of the experiment, all rats received an overdose of sodium pentobarbital and were transcardially perfused with normal saline followed by buffered formalin. The brain stems were removed and stored in formalin for at least one week prior to histological analysis.

Results

The effects of NKA (230 pmol), senktide (30 pmol) and vehicle (1.0  $\mu$ l) on behavior in a novel open field are shown in Fig. 12 and 13. An analysis of the total activity (wall squares entered + center squares entered + rears + head pokes) indicated that there was a significant drug effect [ $F(2,18) = 8.71$ ;  $p < 0.01$ ]. A post-hoc analysis indicated that the activity levels of the animals injected with senktide were significantly greater than that following infusion of vehicle and NKA. Analysis of each component behavior (Fig. 13) demonstrated significant drug effects for wall squares entered [ $F(2,18) = 6.49$ ;  $p < 0.05$ ] and center squares entered [ $F(2,18) = 5.40$ ;  $p < 0.01$ ]. Significant time effects also were observed for wall squares entered [ $F(9, 162) = 14.5$ ;  $p < 0.01$ ], center squares entered [ $F(9,162) = 3.45$ ;

$p < 0.01$ ], head pokes [ $F(9,162) = 3.49$ ;  $p < 0.01$ ], and rears [ $F(9,162) = 8.79$ ;  $p < 0.01$ ].

Post-hoc analysis showed that the significant effects on component behaviors were due to the increases on these measures among the rats which had received infusions of senktide. As can be seen in Fig. 13, the senktide treated group was more active than the other two groups for the entire 30 min period. This is in contrast to the vehicle and NKA infused rats which showed significant reductions in squares entered during the last 12-15 min of the testing period.

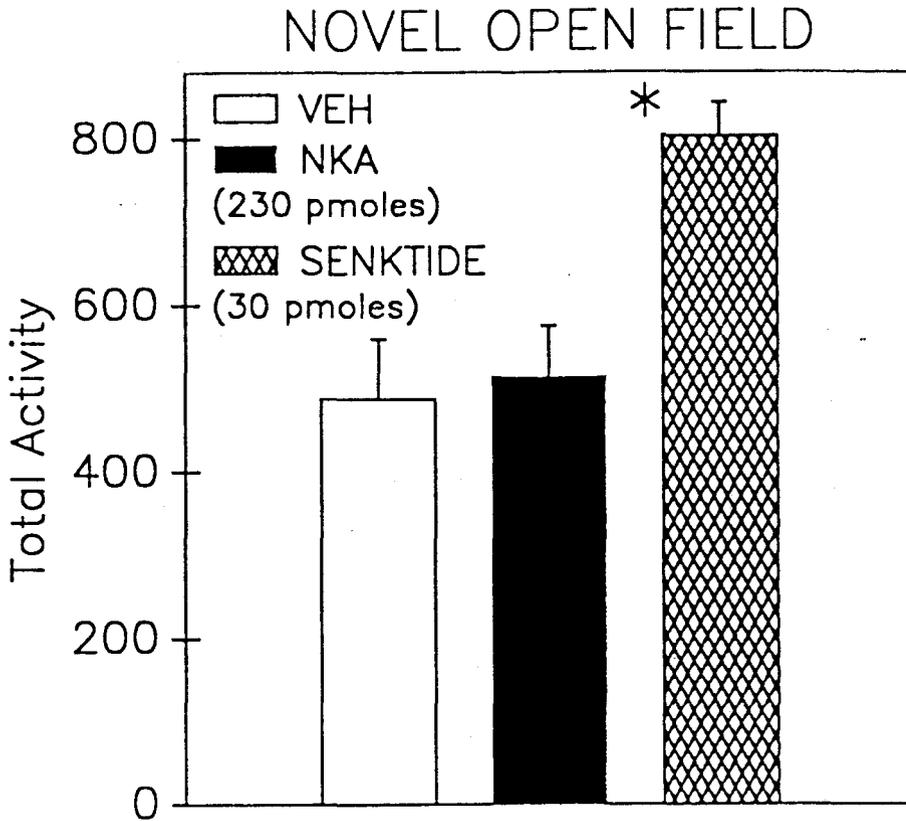


Fig. 12. Neurokinin Effects in a Novel Open Field: Total Activity: Total activity (wall squares + center squares + rears + head pokes) for 30 min following infusion of vehicle, NKA (230 pmol) or senktide (30 pmol). N = 7/9 rats per dose; data represent group means  $\pm$  SEM. \* - significant difference from vehicle infusions,  $p < 0.05$  (ANOVA + Duncan's multiple range test).

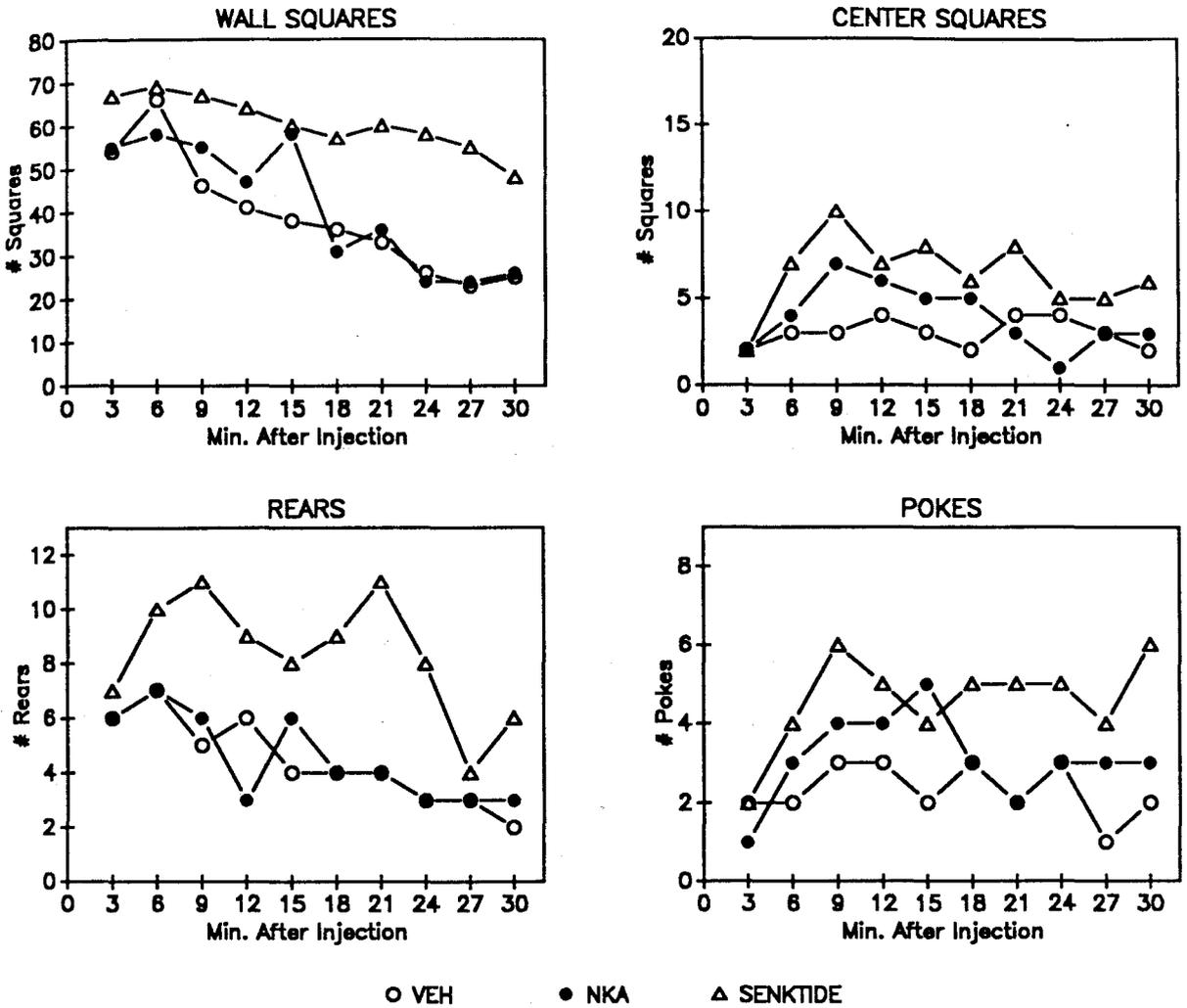


Fig. 13. Neurokinin Effects in a Novel Open Field: Component Behaviors: Number of wall squares, center squares, rears and head pokes per 3 min block for 30 min following infusion of vehicle, NKA (230 pmol) or senktide (30 pmol). N = 7/9 rats per dose; data represent group means; error bars were omitted for clarity.

## Experiment X: Neurokinin-Habituated Open Field

### Procedure

In order to test the effects of habituation on a rat's behavioral response to an intra-MR neurokinin infusion, rats were tested in an open field arena to which they had been adapted. Rats ( $n = 19$ ) were implanted with intra-MR cannulae. Following 7 days recovery, the rats were adapted to the injection procedure and placed in the open field apparatus for 30 min a day for 5 consecutive days. On the sixth day, each rat was removed from its home cage, infused with either vehicle ( $1.0 \mu\text{l}$ ), NKA ( $230 \text{ pmol}$ ) or senktide ( $30 \text{ pmol}$ ), and immediately placed in the open field arena for 30 min. The experiment was conducted over a two week time period with approximately half the rats from each group tested during each week. Following completion of the experiment, the rats were perfused with saline and formalin and their brain stems removed for histological analysis.

### Results

The cannulae placements are shown schematically in Appendix II (Fig. C). An initial analysis comparing the activity measured on the first and last days of habituation indicated a significant reduction in total activity [ $F(1,15) = 37.6$ ;  $p < 0.001$ ], wall squares entered [ $F(1, 15) = 32.5$ ;  $p < 0.001$ ], center squares entered [ $F(1,15) = 31.2$ ;  $p < 0.001$ ], rears [ $F(1,15) = 15.2$ ;  $p < 0.001$ ], and head pokes [ $F(1,15) = 8.63$ ;  $p < 0.01$ ]. This reduction in activity indicates that the animals

habituated to the open field chamber during the five days of adaptation.

The total activity following administration of either vehicle (1.0  $\mu$ l), NKA (230 pmol) or senktide (30 pmol) is shown in Fig. 14. An analysis of the total activity on the test day indicated a significant effect of the neurokinins [ $F(2,16) = 4.41$ ;  $p < 0.05$ ]. Post-hoc comparisons showed that both NKA and senktide significantly elevated activity compared to vehicle infusions.

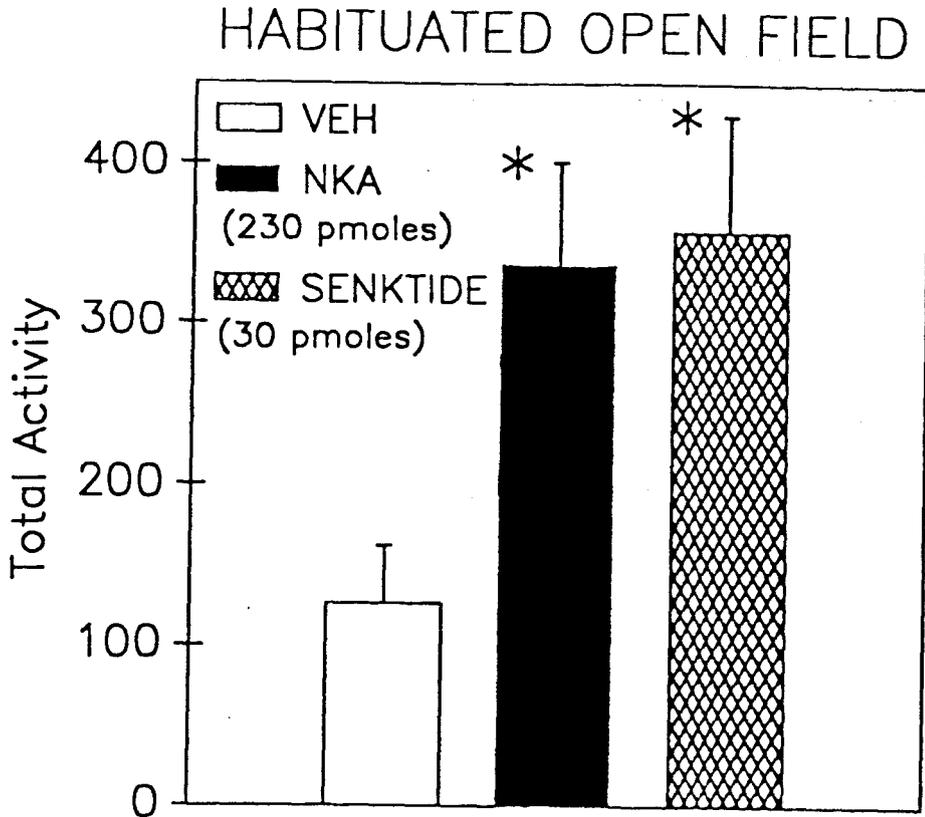


Fig. 14. Neurokinin Effects in an Habituated Open Field: Total Activity: Total activity (wall squares entered + center squares entered + rears + head pokes) for 30 min following infusion of vehicle, NKA (230 pmol) or senktide (30 pmol). N = 7/9 rats per dose; data represent group means  $\pm$  SEM. \* = significant difference from vehicle infusions,  $p < 0.05$  (ANOVA + Duncan's multiple range test).

The effects of vehicle, NKA and senktide on wall and center squares entered, rears and head pokes are shown in Fig. 15. An ANOVA revealed a significant drug effect on wall squares entered [ $F(2,15) = 10.5$ ;  $p < 0.001$ ], center squares entered [ $F(2,15) = 8.38$ ;  $p < 0.01$ ], and rears [ $F(2,15) = 6.52$ ;  $p < 0.01$ ]. Significant time effects and drug x time interactions also were noted for the number of wall squares entered [ $F(7,105) = 11.8$ ;  $p < 0.01$  and  $F(14,105) = 2.82$ ;  $p < 0.01$ , respectively].

The animals which had been infused with either NKA or senktide entered a significantly greater number of squares than the vehicle treated group during the first 15-18 min (Fig. 15). Interestingly, further analysis indicated that the rats infused with NKA entered more center squares and emitted more rears than the other two groups. In contrast, animals injected with senktide tended to enter more wall squares than those injected with NKA over the 24 min test period.

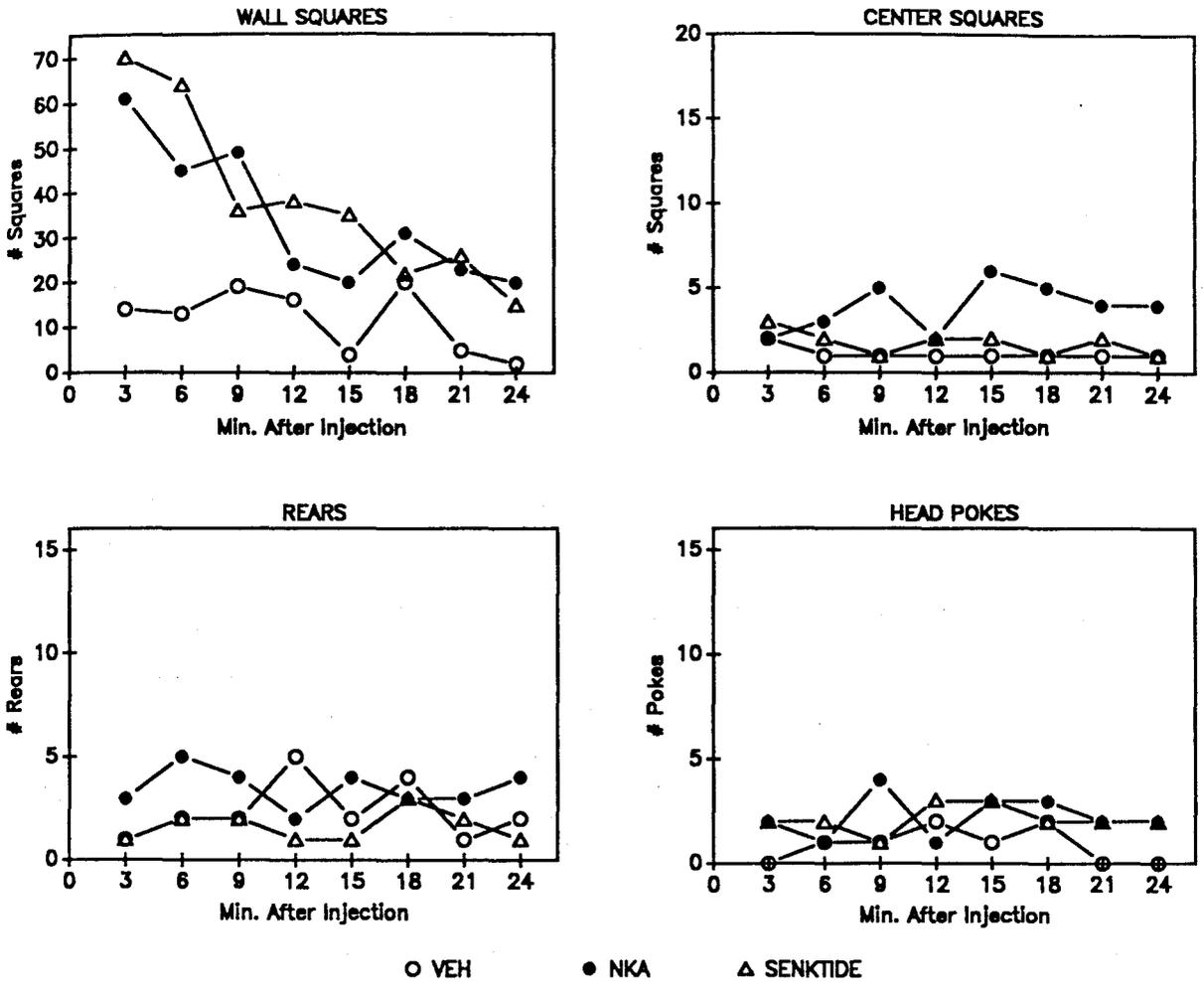


Fig. 15. Neurokinin Effects in an Habituated Open Field; Component Behaviors: Number of wall squares, center squares, rears and head pokes per 3 min block for 30 min following infusion of vehicle, NKA (230 pmol) or senktide (30 pmol). N = 7/9 rats per dose; data represent group means; error bars were omitted for clarity.

## B. Anatomical Studies

### Experiment I: Retrograde Tracing of MR Afferents

The Fluoro-Gold (FG) injection sites are shown in Fig. 16. All were localized within the MR and were approximately 1.0 mm in diameter. The center of each injection site was associated with a small glial scar (approximately 0.25 mm in diameter) which is characteristic of iontophoretic FG administration (Schmued and Fallon, 1986). The number and extent of FG retrogradely-labeled cell bodies were virtually identical in all animals. Fig. 17 schematically portrays the loci of the FG labeled cell bodies observed in a representative animal (FLG-I<sub>2</sub>). The injection site in case FLG-I<sub>2</sub> is pictured in Fig. 18f. Photomicrographs of labeled perikarya in different brain regions are shown in Fig. 18. The description which follows summarizes the results which were consistently obtained in the six rats. Certain exceptions to the labeling pattern will be pointed out.

#### Telencephalon

Retrogradely labeled neurons were found in the following telencephalic areas: frontal and cingulate cortices, septal area, ventral pallidum, and the magnocellular preoptic area. No labeled cells were observed in the caudate-putamen, the nucleus accumbens, the hippocampal formation, or the amygdaloid complex at any anteroposterior level.

### Cortex

As shown in Fig. 17a-g and 18a, a high density of labeled neurons was found throughout the medial aspects of the frontal and cingulate cortices. In two rats (FLG-H<sub>3</sub> and I<sub>1</sub>), a few retrogradely labeled cells (not shown in Fig. 17) were found in the insular cortex. Labeled neurons were not observed in other cortical regions.

### Septal Area

The septal area (Fig. 17d-f) was found to contain a number of retrogradely labeled neurons. Most notable in this region was the high density of labeled neurons in the nucleus of the horizontal limb of the diagonal band (Fig. 18b). Fewer labeled cells were observed in the vertical limb and in the medial septal nucleus. Cells also were encountered within the lateral septal nuclei, more within the intermediate division than in the dorsolateral septum. Adjacent to the septal area, a few labeled neurons were observed in the bed nucleus of the stria terminalis (Fig. 17f).

### Ventral Pallidum, Preoptic Area, and the Substantia Innominata

The ventral pallidum and substantia innominata (Fig. 17e,f) were found to contain a number of retrogradely labeled cells. A large number of FG-labeled cells were observed within the magnocellular preoptic area (Fig. 17f) spread over its medial and lateral subdivisions. It also should be noted that the cells which were found in the horizontal limb of the diagonal band and those found within the ventral pallidum tended to form a continuous band of perikarya.

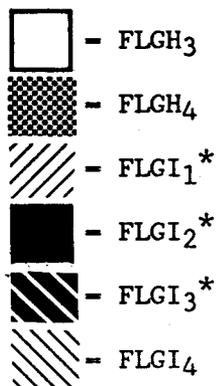
ABBREVIATIONS

3	oculomotor nucleus	f	fornix
4	trochlear nucleus	fi	fimbria
3V	third ventricle	fmi	forceps minor of the corpus callosum
4V	fourth ventricle	fr	fasciculus retroflexus
7	facial nucleus	FR	frontal cortex
7n	facial nerve		
12	hypoglossal nucleus		
aca	anterior commissure, anterior part	g7	genu of the facial nerve
Acb	nucleus accumbens	Gi	gigantocellular reticular nucleus
AHC	central anterior hypothalamic area	GP	globus pallidus
Amy	amygdala	HDB	horizontal limb of the diagonal band of Broca
AOP	anterior olfactory nucleus, posterior	HiF	hippocampal formation
APT	anterior pretectal nucleus	ic	internal capsule
Arc	arcuate nucleus	IC	inferior colliculus
ATg	anterior tegmental nucleus	IO	inferior olive
		IPN	interpeduncular nucleus
BSTM	bed nucleus stria terminalis (medial part)	LC	locus ceruleus
		LDTg	laterodorsal tegmental nucleus
Cg	cingulate cortex	LH	lateral hypothalamus
CG	central gray	LHb	lateral habenular nucleus
CLi	caudal linear raphe nucleus	11	lateral lemniscus
CnF	cuneiform nucleus	LPB	lateral parabrachial nucleus
cp	cerebral peduncle	LPGi	lateral paragigantocellular nucleus
CPu	caudate putamen	LPO	lateral preoptic nucleus
ctg	central tegmental tract	LS	lateral septal nucleus
		LSI	lateral septal nucleus (intermediate part)
Dk	nucleus of Darkschewitsch	LV	lateral ventricle
DMH	dorsomedial hypothalamic nucleus	LVe	lateral vestibular nucleus
DMTg	dorsomedial tegmental area		
DpMe	deep mesencephalic nucleus	m5	motor root of the trigeminal nerve
DR	dorsal raphe nucleus	ME	median eminence
dtb	dorsal tegmental bundle	Med	medial (fastigial) cerebellar nucleus
DTG	dorsal tegmental nucleus of Gudden		
EW	Edinger-Westphal nucleus		

MG	medial geniculate nucleus	RPn	nucleus raphe pontis
MHb	medial habenular nucleus	RRF	retro-rubral field
MiTg	microcellular tegmental nucleus	RtTg	reticulotegmental pontine nucleus
m1	medial lemniscus	RVL	rostroventrolateral reticular nucleus
m1f	medial longitudinal fasciculus	s5	sensory root of the trigeminal nerve
MM	medial mammillary nucleus	scp	superior cerebellar peduncle
Mo5	motor trigeminal nucleus	SFi	septofimbrial nucleus
MPO	medial preoptic nucleus	SI	substantia innominata
MR	median raphe nucleus	sm	stria medullaris
mt	mammillothalamic tract	SNc	substantia nigra (pars compacta)
MVe	medial vestibular nucleus	SNr	substantia nigra (pars reticulata)
MVeV	medial vestibular nucleus (ventral part)	Sol	nucleus of the solitary tract
ox	optic chiasm	sp5	spinal trigeminal tract
pc	posterior commissure	Sp50	spinal trigeminal nucleus (oral part)
PH	posterior hypothalamic area	SPTg	subpeduncular tegmental nucleus
Pir	piriform cortex	Su	superior colliculus
PMV	pre-mammillary nucleus (ventral part)	st	stria terminalis
PN	pontine nuclei	Tu	olfactory tubercle
PnC	pontine reticular nucleus (caudal part)	Tz	trapezoid nucleus
PnO	pontine reticular nucleus (oral part)	VDB	vertical limb of the diagonal band of Broca
PPTg	pedunculo-pontine tegmental nucleus	VLL	ventral nucleus of the lateral lemniscus
PR	prerubral field	VM	ventromedial thalamic nucleus
Pr5DM	principal sensory trigeminal nucleus (dorsomedial part)	VMH	ventromedial hypothalamic nucleus
Pr5VL	principal sensory trigeminal nucleus (ventrolateral part)	VP	ventral pallidum
PrH	prepositus hypoglossal nucleus	VTA	ventral tegmental area
PVN	paraventricular nucleus	VTG	ventral tegmental nucleus of Gudden
py	pyramidal tract	xscp	decussation of the superior cerebellar peduncle
R	red nucleus	ZI	zona incerta
RLi	rostral linear raphe nucleus		
RMg	nucleus raphe magnus		
ROb	nucleus raphe obscurus		
RPa	nucleus raphe pallidus		

Fig. 16. Fluoro-Gold Injection Sites: Fluoro-Gold injection sites in the six rats used in this study. Drawings of coronal sections (designated a-f in upper left of schematic) are adapted from Paxinos and Watson (1986). Numbers at lower left of each drawing indicate distance in mm from the interaural plane. Colchicine-treated rats are indicated below with an asterisk.

LEGEND:



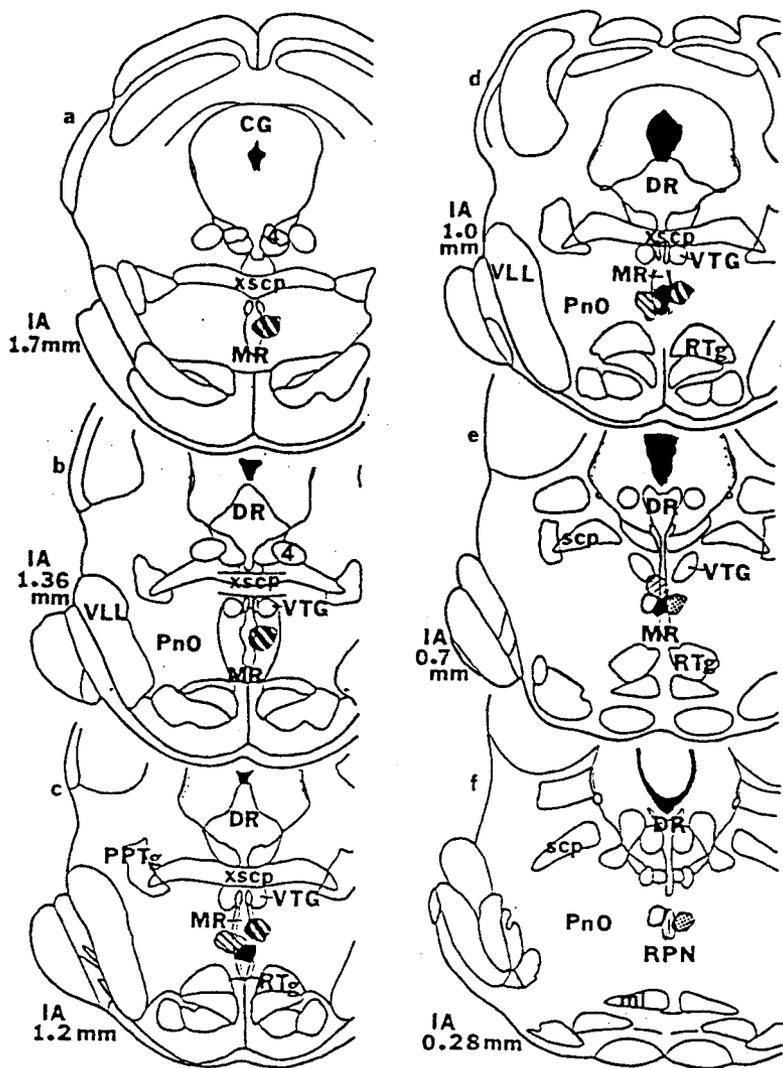
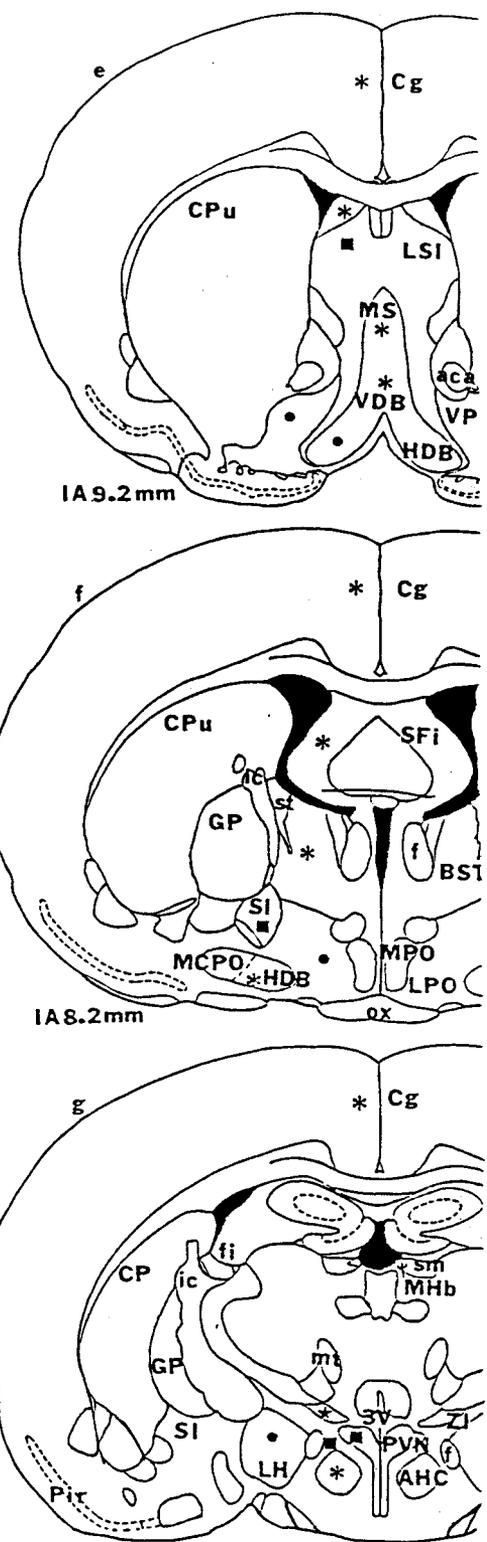
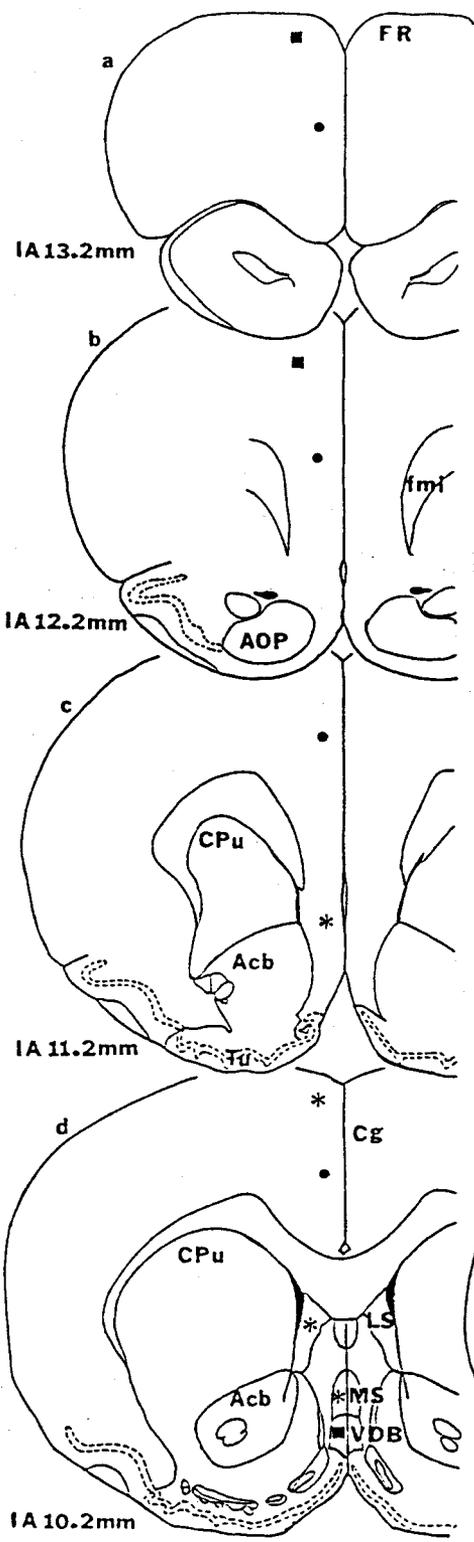
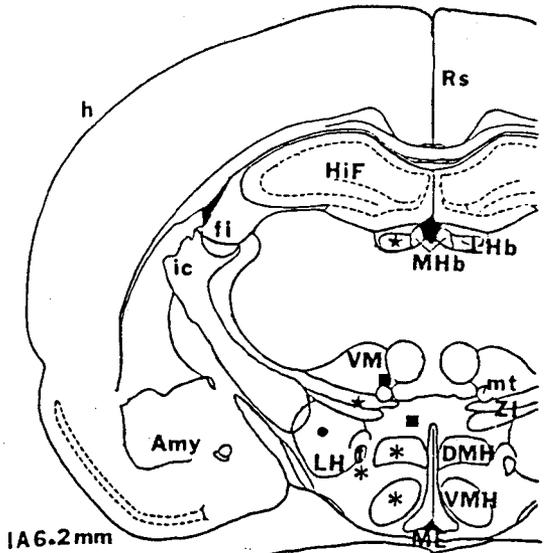


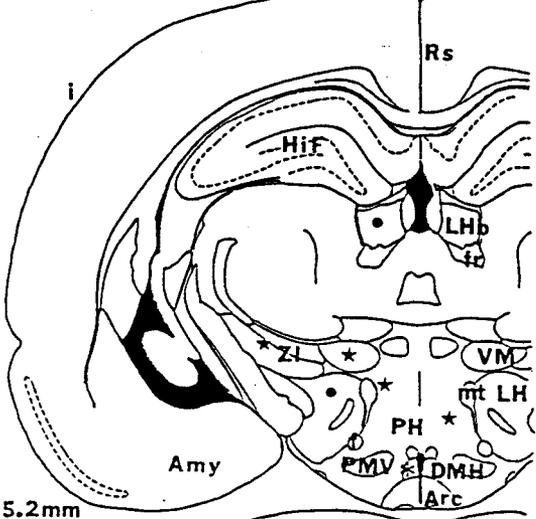
Fig. 17. Localization of Retrogradely Labeled Cells Following MR Fluoro-Gold Infusions: The loci of neurons which were labeled bilaterally following an FG injection into the MR in rat FLG-I<sub>2</sub> (colchicine treated) are plotted on drawings of coronal sections (designated a-t in upper left of schematic) are adapted from Paxinos and Watson (1986). Numbers at lower left of each drawing indicate distance in mm from the interaural plane. The injection site is indicated by the blackened area in the MR (see sections m-o) and illustrated in Fig. 16 c-e and 18 f.

LEGEND: Asterisk - 1 - 5 cells  
Square - 6 - 10 cells  
Star - 11 - 20 cells  
Circle - > 20 cells

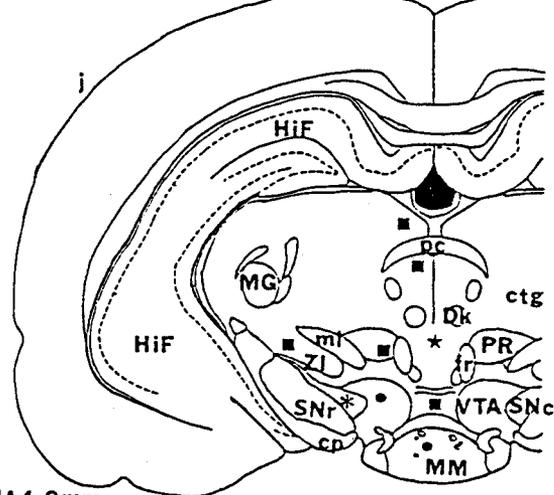




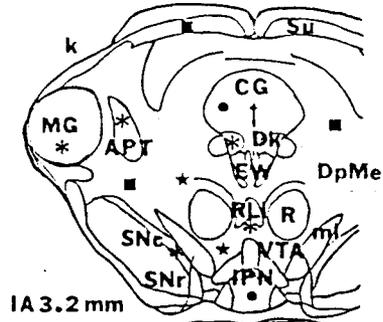
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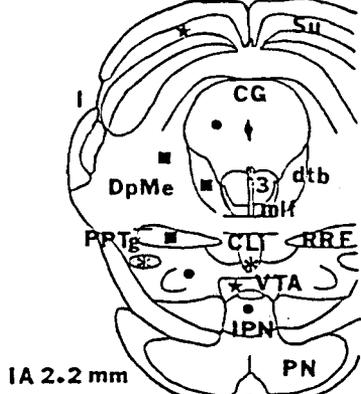
IA 5.2 mm



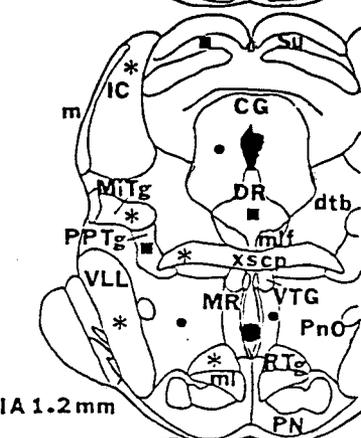
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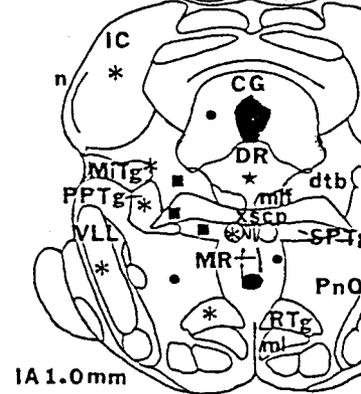
IA 3.2 mm



IA 2.2 mm



IA 1.2 mm



IA 1.0 mm

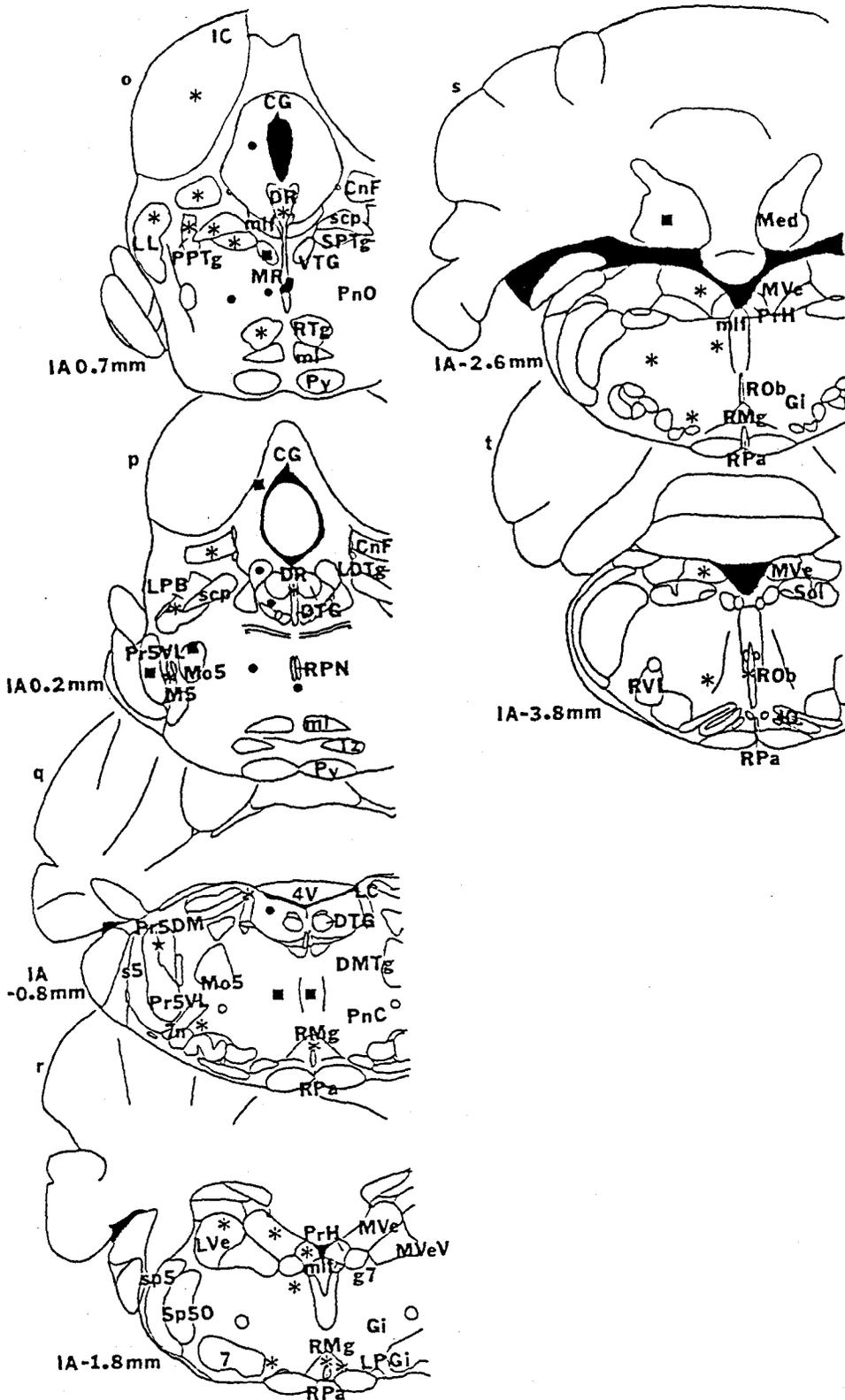
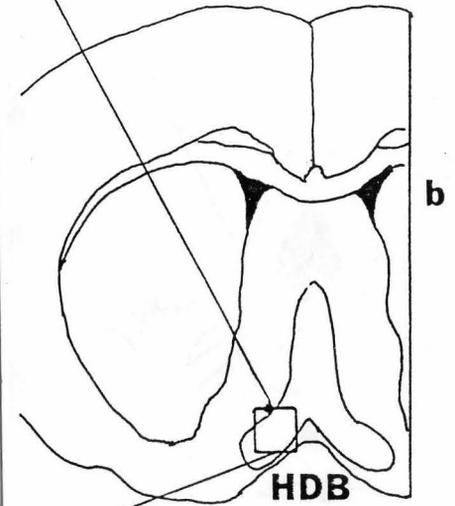
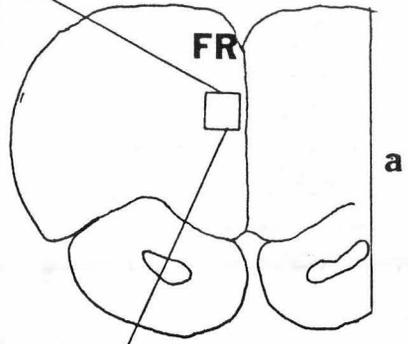
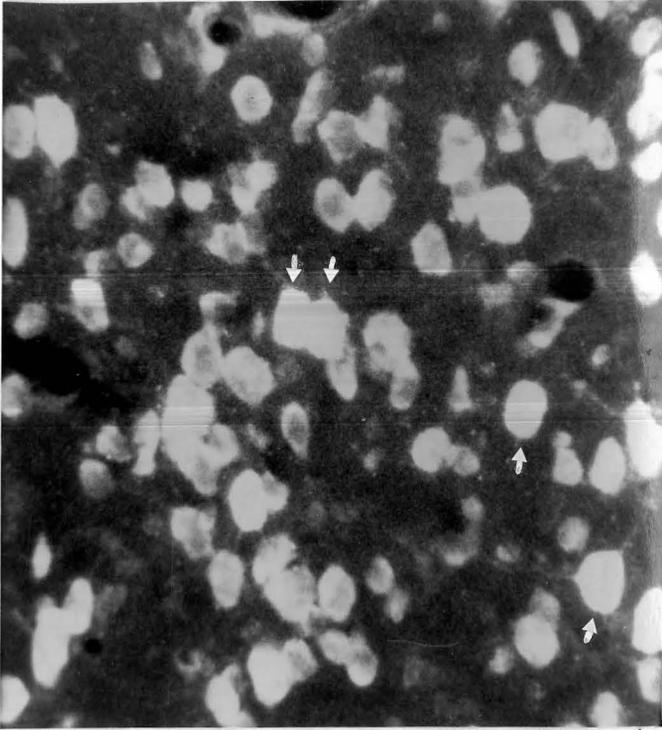
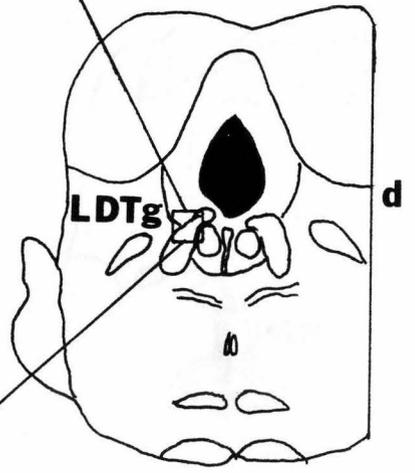
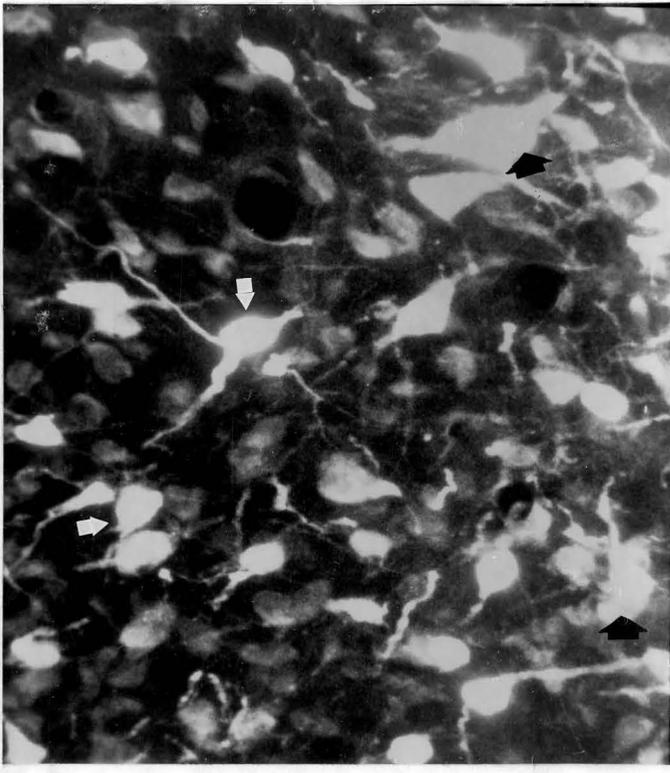
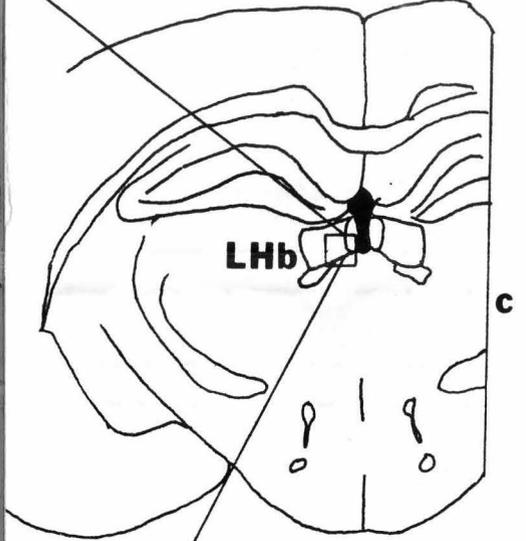
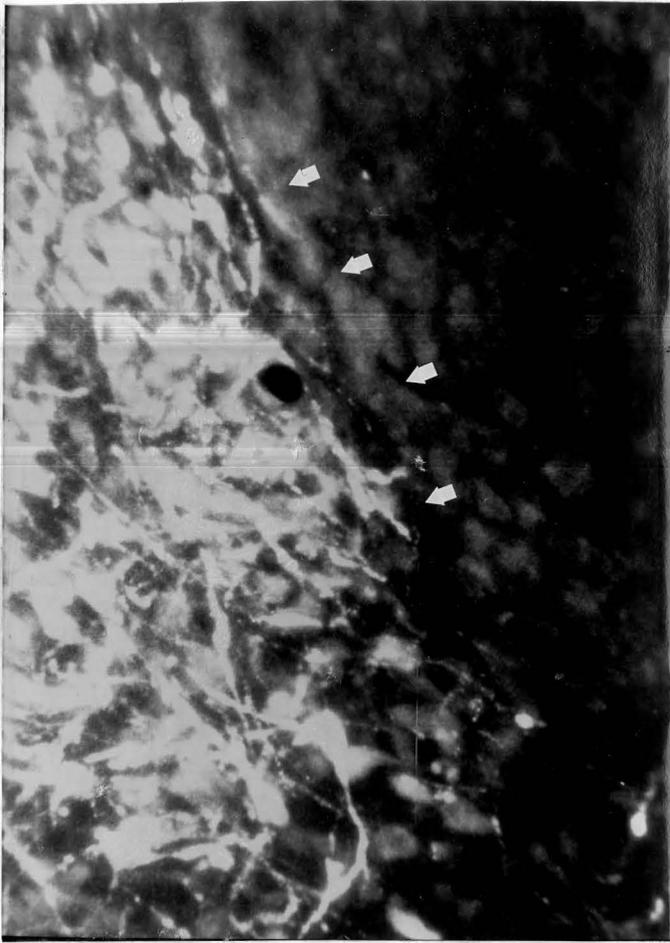
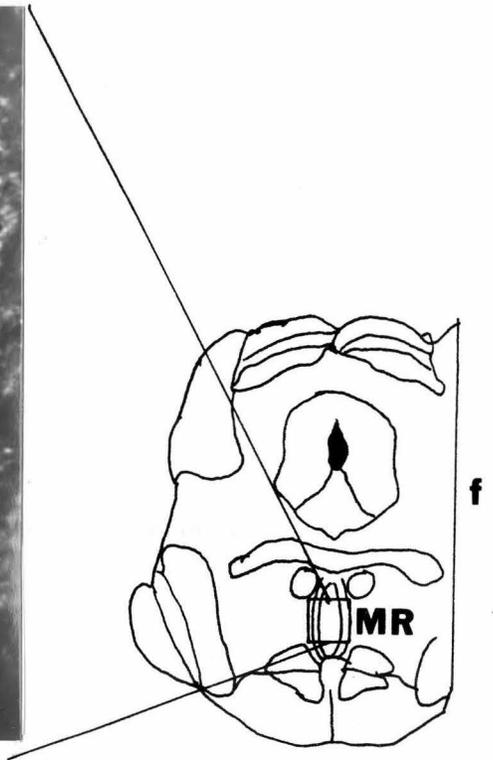
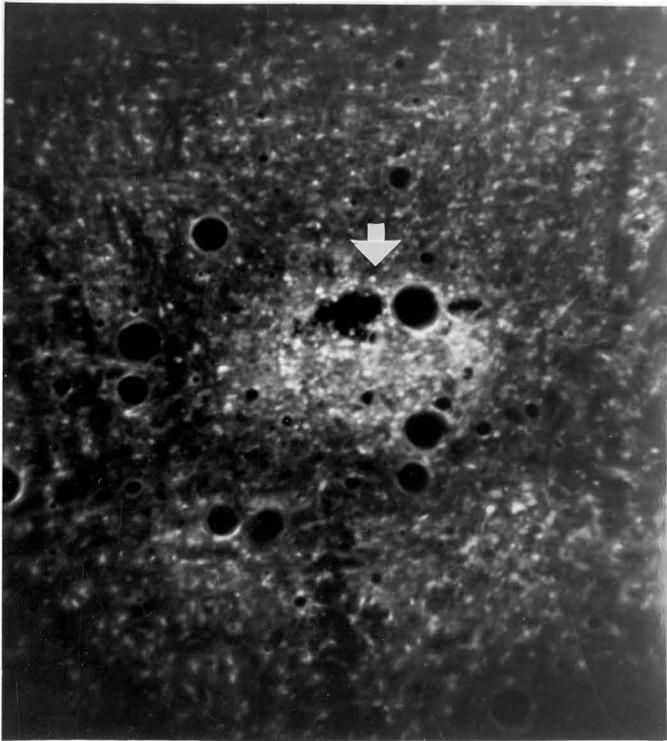
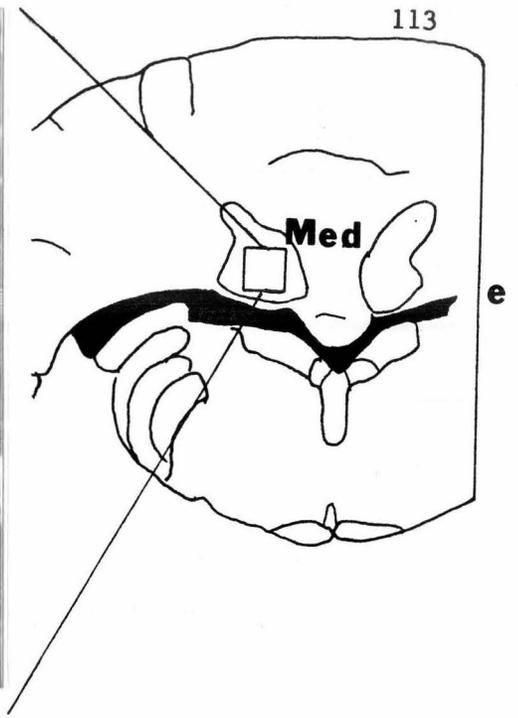
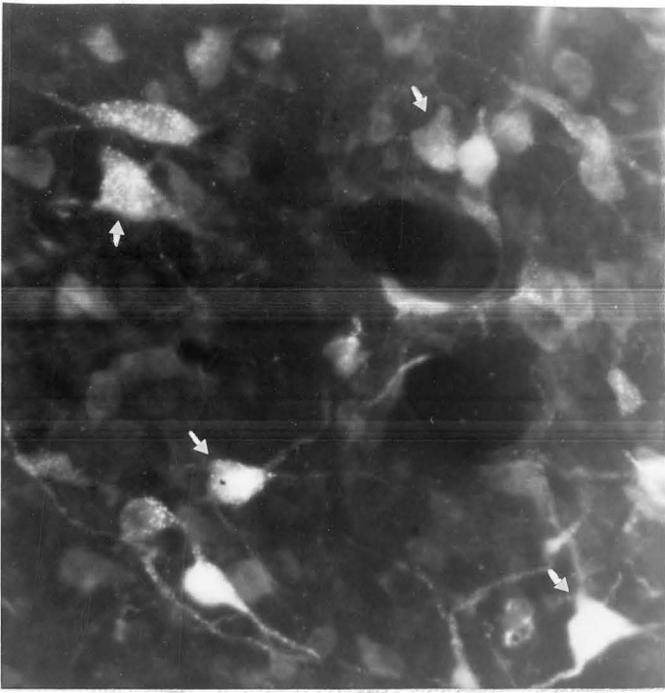


Fig. 18. Representative Photographs of Fluoro-Gold Labeled Neurons: Photomicrographs (x250; except in f where magnification was x160) of: a) medial frontal cortex; b) horizontal limb of the diagonal band of Broca; c) lateral habenular nucleus; d) laterodorsal tegmental nucleus; e) medial nucleus of the cerebellum; and, f) injection site. Representative Fluoro-Gold labeled cells in each section are indicated with arrows. Drawings of coronal sections are adapted from Paxinos and Watson (1986).







## Diencephalon

Fluoro-Gold labeled neurons were found be distributed discretely within the following diencephalic regions: the lateral habenular nucleus, the ventromedial thalamic nucleus, and the hypothalamus.

### Epithalamus

The diencephalic region which contained the greatest number of retrogradely filled cells was the lateral habenular nucleus (Fig. 17h,i; Fig. 18c). No FG-labeled neurons were observed in the adjacent medial habenular nucleus. An occasional labeled cell was found embedded within the stria medullaris, just dorsal to the habenular complex.

### Dorsal Thalamus

The ventromedial nucleus was the only dorsal thalamic nucleus found to contain retrogradely labeled neurons (Fig. 17h-j). Most of these cells were clustered around the mammillothalamic tract. This group of labeled neurons also seemed to be continuous with those found more posteriorly in the prerubral fields.

### Subthalamus

A number of FG-labeled cells were encountered within the zona incerta (Fig. 17g-j) and the area immediately surrounding the mammillothalamic tract.

## Hypothalamus

A number of areas within the hypothalamus (Fig. 17g-j) were found to contain retrogradely labeled neurons. These included the anterior, lateral, ventromedial, dorsomedial, paraventricular, and mammillary nuclei. The densest number of labeled cells were localized throughout the lateral hypothalamic area and the mammillary region. A number of cells were found surrounding the fornix.

## Mesencephalon

FG filled neurons were observed in the region just dorsal and lateral to the posterior commissure (Fig. 17j). Cells were observed ventral to the posterior commissure, immediately lateral to the third ventricle and dorsal to the nucleus of Darkschewitsch. This latter nucleus also was found to contain a few labeled neurons (Fig. 17k).

The ventral tegmental area (Fig. 17j-l) contained a large number of labeled cells. A few FG-filled neurons also were found in the substantia nigra, pars compacta (Fig. 17j,k).

A large number of retrogradely labeled cells were found more caudally in the midbrain (Fig. 17i-o), especially in the central gray, the superior colliculus, the deep mesencephalic nucleus lateral to the red nucleus, and the interpeduncular nucleus. A few labeled cells also were found in the medial geniculate, the anterior pretectal nuclei, and the inferior colliculus.

The rostral rubral field contained FG filled neurons, as did the rostral and caudal linear raphe nuclei. The dorsal raphe (DR) nucleus contained FG-labeled neurons as did all portions of the MR surrounding

the injection site. A few labeled cells were seen in the ventral tegmental nucleus of Gudden.

### Metencephalon

At pontine levels a large number of retrogradely labeled cells were found in the nucleus raphe pontis, and in both the oral and caudal parts of the pontine reticular nucleus (Fig. 17m-q). Scattered cells could be seen in the ventral nucleus of the lateral lemniscus, and in the reticulopontine tegmental, and subpedunculotegmental nuclei. Some FG-filled neurons were found in the cuneiform nucleus and in the pedunculopontine and adjacent microcellular tegmental nuclei.

More caudally, the laterodorsal tegmental nucleus (LDTg) contained a prominent number of FG-filled cells (Fig. 17p,q; Fig 18d). Labelled cells also were found in the motor, motor nucleus, and most notably, in the principal sensory nucleus (ventrolateral part) of the trigeminal complex (Fig. 17p,q). In colchicine treated rats, these labeled neurons were observed to send dendritic processes into the adjacent dorsal tegmental nucleus of Gudden and trigeminal nuclei.

### Cerebellum

A number of retrogradely labeled cells were detected within the medial nucleus of the cerebellum (Fig. 17s; Fig. 18e). This was the only cerebellar region found to contain FG-filled cells.

### Myelencephalon

At medullary levels (Fig. 17q-t), scattered FG-filled neurons were detected in the nucleus raphe magnus, pallidus, and obscurus; the medial and lateral vestibular nuclei; and the prepositus hypoglossal nucleus. Within the medullary reticular formation, labeled cells were found in the gigantocellular and lateral paragigantocellular nuclei.

### Lower Medulla and Spinal Cord

FG-labeling in the lower medulla and spinal cord was investigated in only one rat (FLG-I<sub>3</sub>). A few (1-3/nucleus) labeled cells were found in: the rostroventrolateral reticular nucleus; the dorsal and ventral medullary reticular field; the nucleus of the solitary tract (bordering the dorsal motor nucleus of the vagus); the dorsal and paramedian reticular nuclei; the lateral reticular nucleus; layers 3, 5, 7, 8 of the cervical cord; the central cervical nucleus; layers 2 and 8 of the thoracic cord; and, along the border of layers 4 and 10 of the lumbar spinal cord.

## Experiment II: Neurokinin Fiber Staining

Representative sections depicting immunocytochemical staining for neurokinin fibers are shown in Fig. 19. SP- and neurokinin-like immunoreactivity (SPLI and NKLI, respectively) were found within the MR, the DR, the interpeduncular nucleus, and within the principal sensory trigeminal nucleus. Results with the anti-SP and anti-NKA antisera were virtually identical.

### Controls

In order to test the specificity of the primary antibodies used, sections through the interpeduncular nucleus, which contains a very dense neurokinin fiber network, were incubated with primary antibody and with primary antibody which had been preabsorbed with the respective antigen (SP and NKA; 100  $\mu\text{g/ml}$  of diluted antibody). Fig. 20 shows the immunocytochemical staining obtained with the unabsorbed antisera (a) and the absence of staining with the preabsorbed primary antisera (b).

Previous cross-absorption studies with the SP2 and NKA2 primary antibodies indicated that the immunoreactivity was still observed following preabsorption of the SP2 antiserum with NKA or NKB. Preabsorption of the NKA2 antibody with SP did not affect the fiber labeling, but preabsorption with NKB did eliminate it (Brodin et al., 1986). Cross-absorption studies with the commercially obtained anti-SP antibody indicated a 5-10% cross-reactivity with NKA and no apparent cross-reactivity with NKB (Dr. Mary Frick, Incstar, Inc., personal communication, 1988). Substitution of the primary antibodies with diluent (omit controls), eliminated all staining.

Fig. 19. Neurokinin-Like Immunoreactive Fibers: Photomicrographs (x250) of sections through a) the median raphe nucleus (MR), and, b) the principal sensory trigeminal nucleus (Pr5VL). The MR section was incubated with the rabbit anti-NKA primary antibody, whereas the Pr5VL section was incubated with the rabbit anti-SP antiserum.

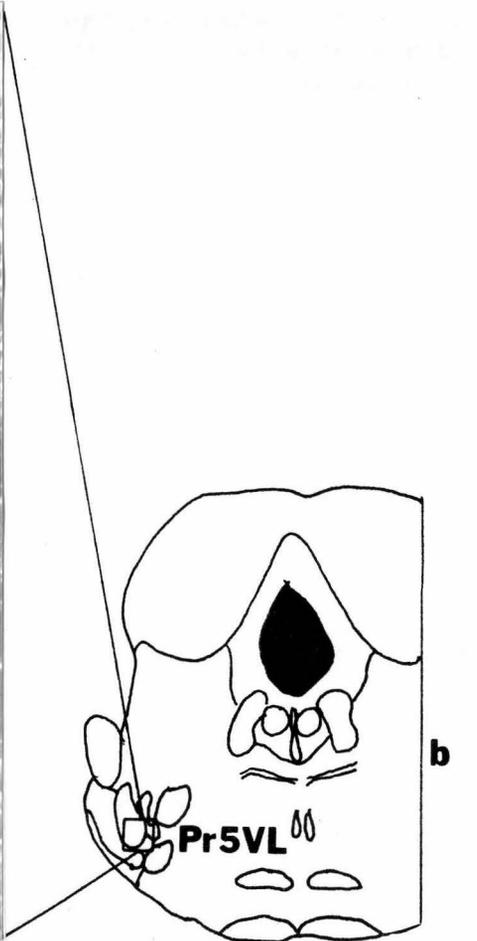
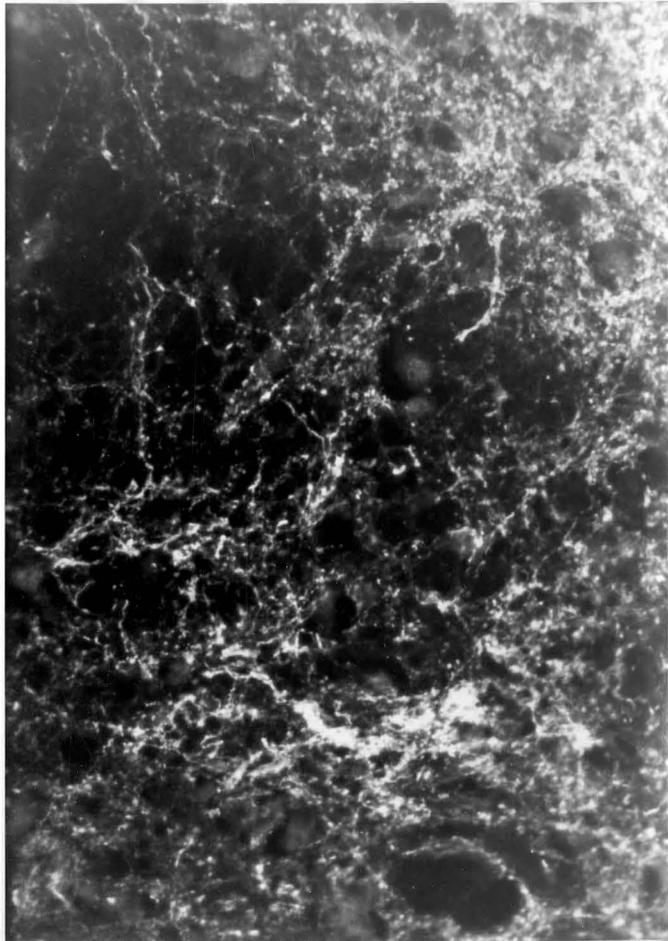
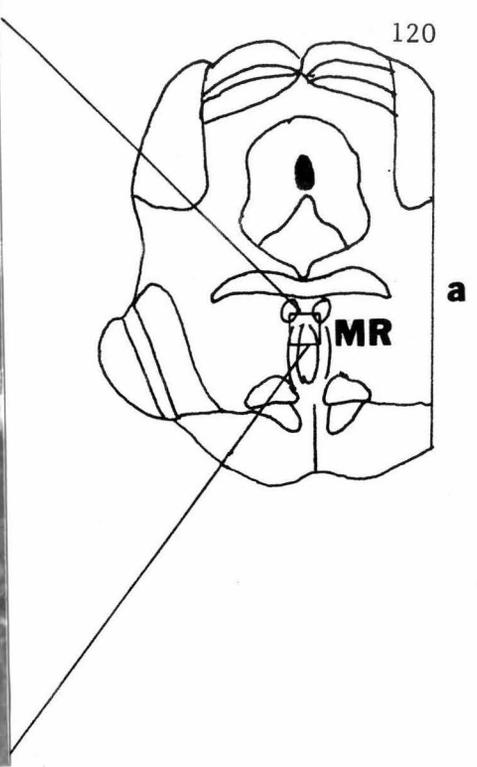
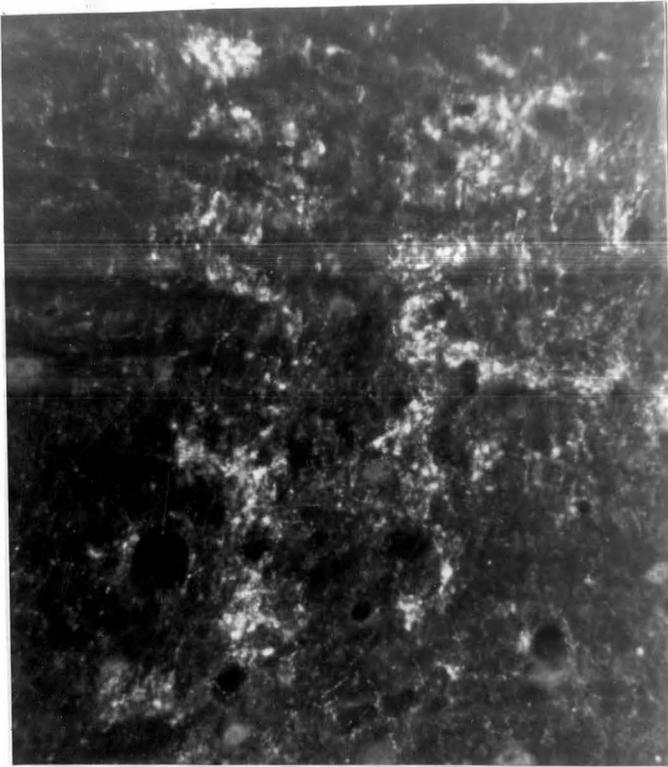
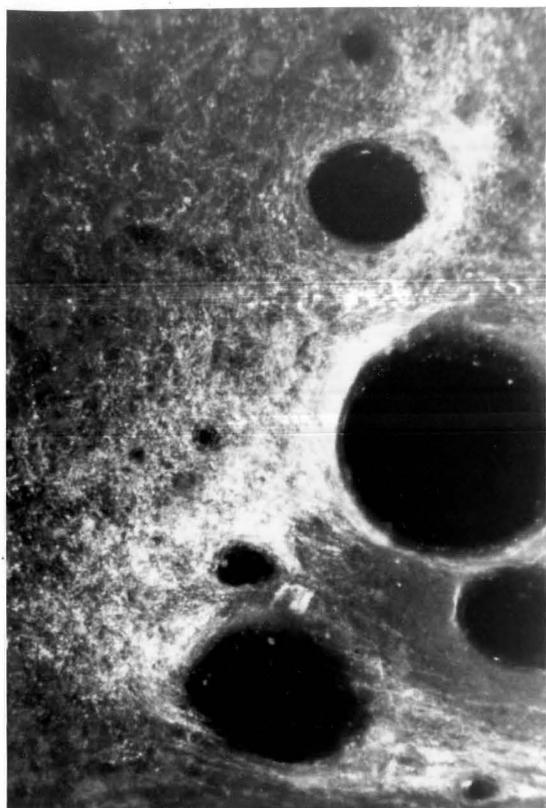
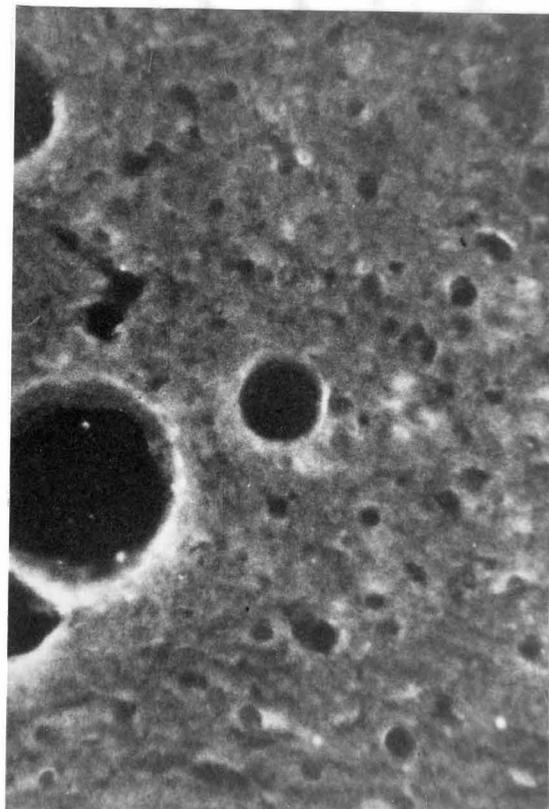
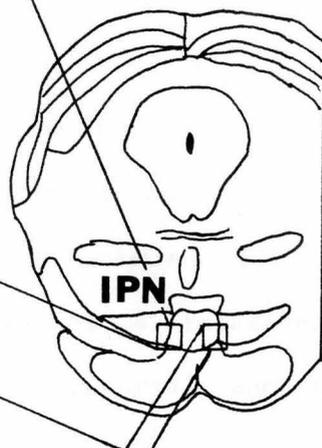


Fig. 20. Preabsorbed Control: Photomicrographs (x250) of sections through the interpeduncular nucleus (IPN) stained with a rabbit anti-SP antibody without preabsorption (a), and following absorption overnight with SP.



**a**



**b**

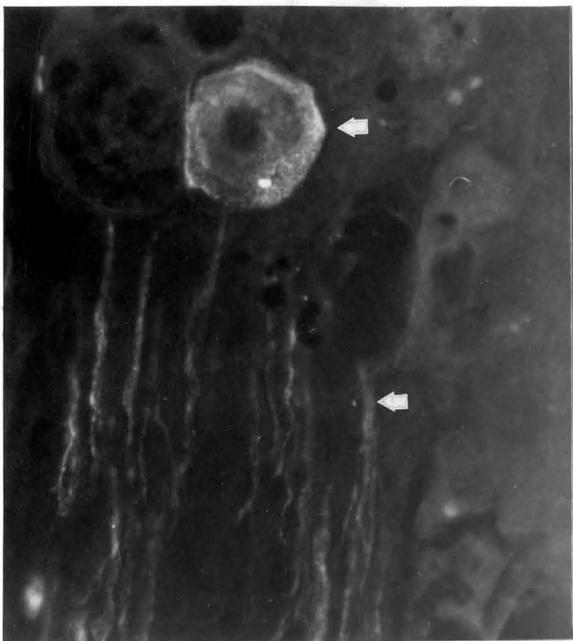
### Experiment III: Neurokinin Perikarya Staining

In order to determine whether our immunocytochemical procedures would enable us to visualize neurokinin immunoreactive cell bodies, sections through the rat dorsal root ganglia were processed for study. Ganglia were obtained from an untreated and an intrathecally colchicine pretreated rat. Fig. 21 shows the localization of both SPLI and NKLI within the dorsal root ganglia.

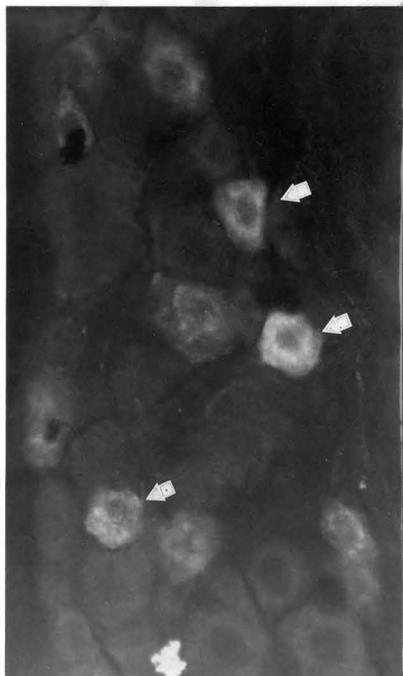
The results of the immunocytochemical staining of neurokinin cell bodies in the CNS are shown in Fig. 22. SPLI and NKLI-perikarya were observed in a number of regions previously reported to contain SP or NK reactive perikarya. The best staining was found in the following regions: the rostral central gray, the interpeduncular nucleus, the dorsal raphe nucleus, and the laterodorsal tegmental nucleus. Fig. 22 shows immunoreactive cells in the medial habenular nucleus, interpeduncular nucleus, the rostral central gray, and the laterodorsal tegmental nucleus. Occasional SPLI and NKLI cells also were encountered within the septum, the paraventricular nucleus, the caudate-putamen, and the lateral hypothalamus.

Fig. 21. Neurokinin-Like Immunoreactivity in Dorsal Root Ganglia: Photomicrographs (x250) of SP-immunoreactive cells and fibers in the dorsal root ganglia following incubation with the rabbit anti-SP antibody in a colchicine-treated (a), and an untreated rat (b). Photomicrograph (x250) of an NKA-immunoreactive cell following incubation with the rabbit anti-NKA antiserum is shown in (c).

**a**

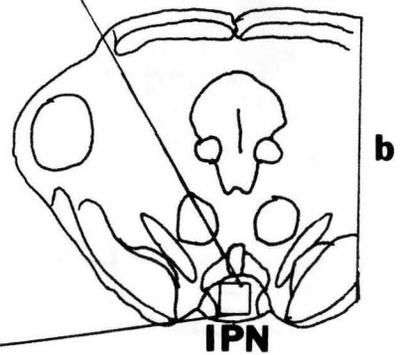
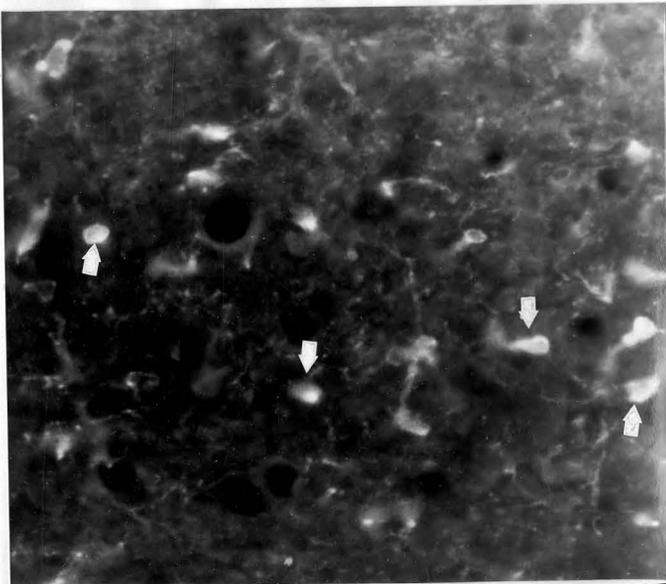
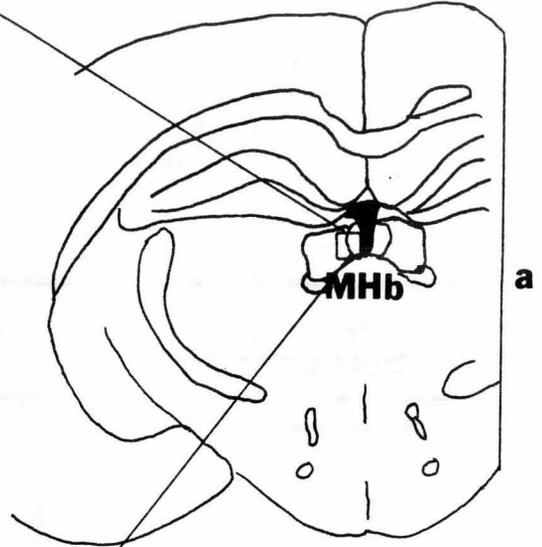
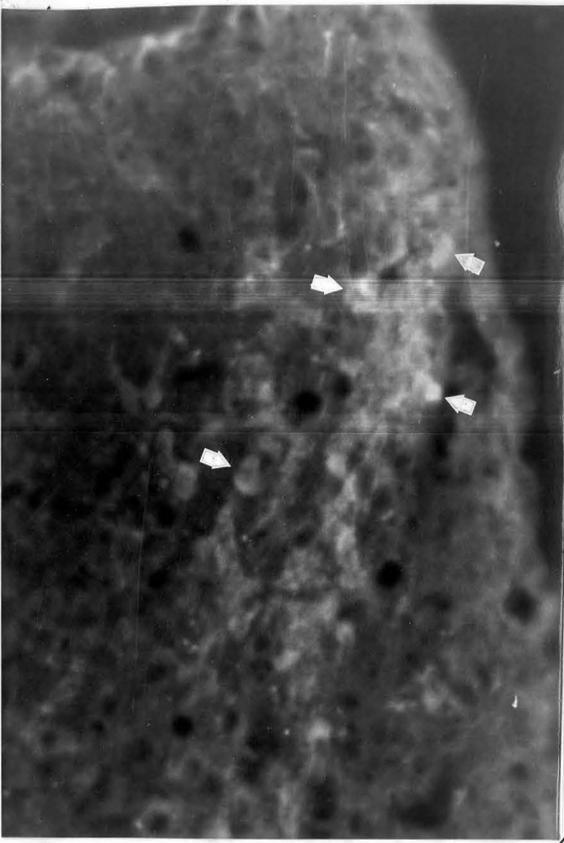


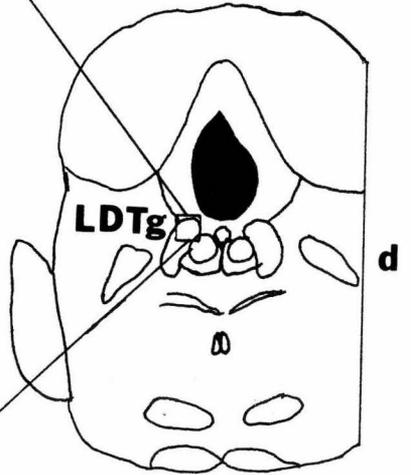
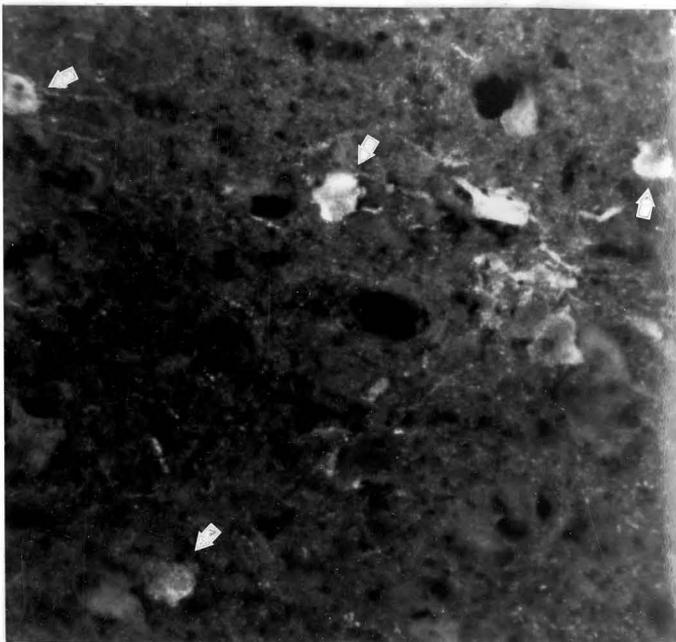
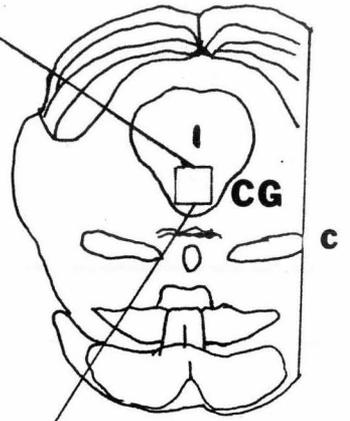
**b**



**c**

Fig. 22 (a-d). Neurokinin-Like Immunoreactive Perikarya: Photomicrographs (x160 in a and b; x250 in c and d) of SP-immunoreactive cells, from colchicine-treated rats, following incubation of the tissue sections with the rabbit anti-SP antibody. Sections are through: a) the medial habenular nucleus (MHb); b) the interpeduncular nucleus (IPN); c) the rostral central gray (CG); and, d) the laterodorsal tegmental nucleus (LDTg).

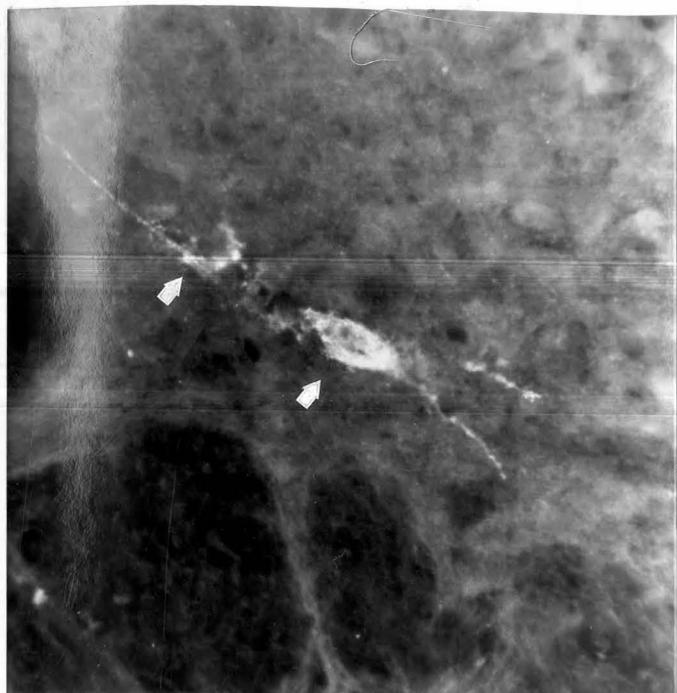




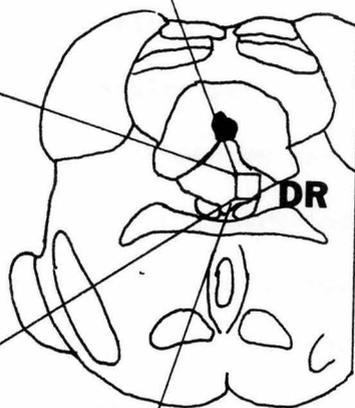
Combined Fluoro-Gold and Neurokinin Immunocytochemistry

The brains from two rats which had received an infusion of FG into the MR, and colchicine treatment one week later, were processed for immunocytochemistry. Sections were saved through regions which had been found to give adequate staining for neurokinin perikarya: the medial habenular nucleus, the interpeduncular nucleus, the dorsal raphe, the rostral central gray, and the laterodorsal tegmental nucleus. With the exception of the medial habenular nucleus, all of these regions were found to be the source of MR afferents. By using the appropriate filter combination, separate populations of either FG or FITC labeled cells could be discerned in each region. There were no clear cut cases of doubled-labeled neurons. In one case, initial viewing of a section through the laterodorsal tegmental nucleus suggested the possibility of a doubled-labeled cell which was not verified upon subsequent viewing. Although it is possible that the fluorescence may have faded, staining of the laterodorsal tegmentum in another animal did not reveal any double-labeled neurons. Fig. 22 (e,f) shows a section through the dorsal raphe photographed using the filter combinations for visualization of FG and FITC.

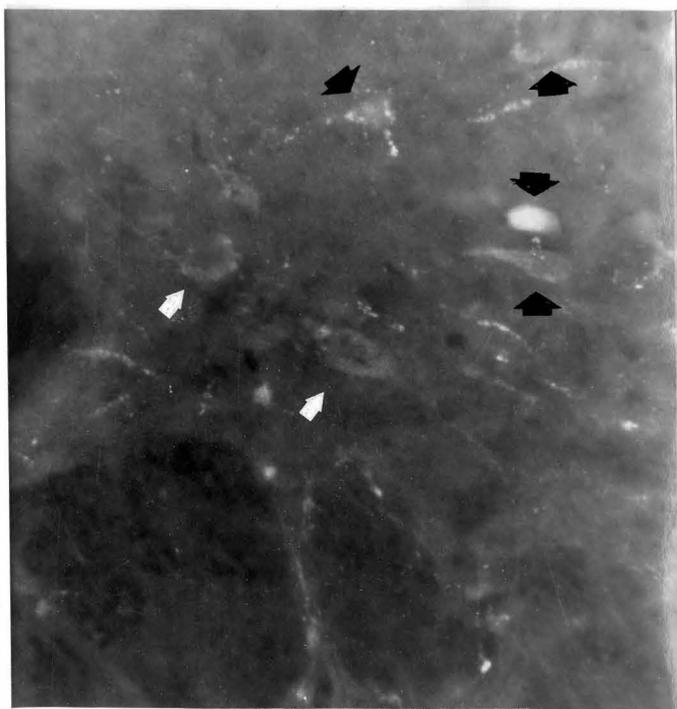
Fig. 22 (e,f). Combined Retrograde Labeling and Neurokinin Immunocytochemistry: Section through the dorsal raphe (DR) showing SP-immunoreactive and FG-filled neurons. Section was photographed (x250) to visualize FITC (e) and FG-labeled cells (f). Note that the FG-labeled cells (black arrows in f) are not visible in e. Although some "bleed-through" of the fluorescence can be seen, the FITC labeled cells (white arrows in e) are not visible in f.



**e**



**DR**



**f**

## CHAPTER V

### DISCUSSION

The purpose of this dissertation project was to investigate the functional relationships between neurokinin and 5-HT neuronal processes within the median raphe nucleus (MR) of the rat midbrain. The first series of experiments studied the behavioral effects following tachykinin injections into the MR. It was found that activation of two different neurokinin receptors (NK-2 and NK-3) could elicit locomotor hyperactivity, and that agonists (NKB and senktide) selective for the NK-3 site were ten times more potent than agonists (NKA) selective for the NK-2 site. Lesions made with 5,7-DHT confirmed that the hyperactivity produced by neurokinin injections into the MR depend on the integrity of MR 5-HT neurons. The hyperkinesis produced by activation of NK-2, but not NK-3, sites may be mediated, at least in part, by 5-HT projections to the forebrain. Analyses of animals' behavior in an open field suggested, furthermore, that the behavioral changes elicited by MR injections of the neurokinins depend on the degree of familiarity or novelty of the environment.

Using a retrograde tracing technique and immunocytochemistry, the second series of experiments examined the origin of the neurokinin innervation to the MR. The MR was found to receive afferent projections from many discretely localized brain structures. Immunocytochemical studies confirmed that the MR contains SP-like and neurokinin-like

immunoreactive fibers. Analyses of the combined immunocytochemical and retrograde labeling data suggested, furthermore, that the forebrain is the source of the neurokinin innervation to the MR.

### A. Behavioral Studies

#### Dose-Response Analysis of Intra-MR Neurokinin Infusions

The first series of experiments were undertaken in order to determine the relative rank order of potency for intra-MR injected tachykinins to induce locomotor hyperactivity. The dose-response analyses suggest that hyperkinesis can be elicited by activation of two different neurokinin receptors. At the NK-2 site, NKA was more potent than SP and the non-mammalian tachykinins, while at the NK-3 site, senktide was more potent than DiMe-C7. Importantly, the hyperactivity produced by intra-raphé infusions of neurokinin B (3.0 pmol) was equivalent to that produced by an equimolar dose of senktide. The results suggest that activation of NK-2 and NK-3 receptors in the MR lead to elevations in the level of behavioral arousal. Intra-MR neurokinin-induced hyperactivity, moreover, may provide a novel in-vivo model for studying NK-2 and NK-3 receptor activation in the central nervous system of the rat.

Stoessl et al. (1987 a,b) have reported that peripheral or intracisternal infusions of senktide produce the classical "serotonin-syndrome" (head twitching, forepaw treading, hind limb splaying) in both rats and mice. The experimental procedure used for measuring LMA in the present study (a darkened photocell chamber) does not permit one to

differentiate photocell breaks produced by locomotion from those produced by stereotypic movements. However, the analyses of the rats' behavior in an open field following the intra-MR injection of DiMe-C7, NKA and senktide indicated that the animals were not engaged in any of these "serotonin-mediated" or other types of stereotypies. In contrast, the animals exhibited an increase in a variety of exploratory behaviors, such as walking, rearing, sniffing, and head poking.

The non-mammalian tachykinins, ELE, KAS, and PHY, all produced increases in LMA, although ELE was the most potent and the most efficacious. Binding studies indicate that radiolabeled ELE labels NK-3 binding sites in rat brain (Bergstrom et al., 1987; Glowinski et al., 1987). Isolated peripheral tissue preparations, such as the rat portal vein, are sensitive to low doses of both NKB and ELE (Drapeau et al., 1987; Mastrangelo et al., 1986). However, this assay also is sensitive to KAS, suggesting that this in-vitro preparation does not necessarily reflect binding potencies or pharmacodynamic interactions within the CNS.

Senktide was the most potent (minimum effective dose: 3.0 pmol) and the most efficacious neurokinin analogue to elicit hyperactivity. The relative potencies of the neurokinin agonists were: senktide (= NKB) > DiMe-C7, and NKA >> SP. Radiolabeled senktide was developed as a selective NKB agonist (Laufer et al., 1986a; Wormser et al., 1986), and binds to NK-3 sites in rat brain. Thus, the data suggest that the intra-raphé effects of senktide on LMA are mediated by NK-3 receptors.

Infusions of 3.0 pmol NKB, equimolar to the lowest dose of senktide which produced a significant increase in LMA, induced locomotor

hyperactivity which was equivalent to that of senktide. Since it was necessary to dissolve NKB in a formic acid vehicle, the experiment was limited to a single injection. The morphology of the cells immediately surrounding the cannula tips in rats which received either the formic acid vehicle or NKB infusions was identical to that seen in rats infused with other compounds. However, in a few instances, a thin "halo" (approximately 0.2 mm thick), devoid of perikarya, was observed surrounding the cannula tips. Nevertheless, the formic acid vehicle infusions alone did not affect LMA (see Fig. 8). These results corroborate those obtained with senktide.

Activation of an NK-2 or NK-3 receptor in the MR of the rat leads to an increase in behavioral arousal. Neurokinin-like fibers (but not cell bodies) and receptors have been observed in the MR. The effects of the neurokinins, in contrast to those of intra-MR infused muscimol, depend upon intact 5-HT neurons (Paris and Lorens, 1987 a,b; and present study). This suggests that NKA and NKB projections to the raphe modulate 5-HT neuronal activity.

Although MR NK-2 receptor activation leads to locomotor hyperactivity, it should be noted that the presence of NK-2 receptors in the CNS has been questioned (Glowinski et al., 1987; Saffroy et al., 1988). It must be emphasized, furthermore, that without the availability of non-neurotoxic neurokinin antagonists, one can only utilize the relative potencies of the various agonists to identify the possible receptor(s) involved.

MR and Medial Forebrain Bundle 5,7-DHT Lesions

Infusion of the SP analogue, DiMe-C7, into the MR produces dose-dependent increases in LMA which can be blocked by pretreatment with the tryptophan hydroxylase inhibitor, PCPA, and by MR 5,7-DHT lesions (Paris and Lorens, 1987 a,b). One of the objectives of the present study was to determine if 5,7-DHT lesions of the MR would similarly block the effects of intra-raphe infusions of senktide and NKA. Doses which previously were determined to produce equivalent increases in LMA (see Fig. 4), were administered to rats two weeks following 5,7-DHT lesions of the MR. These lesions completely blocked the hyperactivity produced by the intra-raphe neurokinin infusions. In order to test whether 5-HT projections to the forebrain mediate the LMA effects of intra-MR infused neurokinins, 5,7-DHT was injected bilaterally into the medial forebrain bundle (MFB), anterior to the DA cell bodies of the rostroventral midbrain tegmentum. These lesions, in contrast to the 5,7-DHT lesions of the MR, did not block the hyperactivity produced by the intra-raphe injections of senktide and DiMe-C7, although they appeared to attenuate the hyperactivity produced by NKA. These results indicate that intra-raphe neurokinin induced hyperactivity produced by activation of NK-3 receptors depends on the integrity of 5-HT neurons in the MR, but not on the 5-HT fibers which ascend through the MFB to innervate the forebrain. On the other hand, NK-2 receptor mediated hyperactivity may depend in part upon 5-HT fibers which innervate forebrain regions.

Since the MFB 5,7-DHT lesions were placed in the lateral hypothalamus, rostral to the DA cell bodies in the ventral tegmental area

(VTA) and substantia nigra (SN), it is possible that 5-HT projections to these structures are critical for intra-MR neurokinin induced effects on LMA. The DA neurons in the substantia nigra (A9) and rostro-ventromedial mesencephalon (A10), and the forebrain regions they innervate, have been implicated in the regulation of motor function (Le Moal et al., 1975; Koob et al., 1981; Bannon and Roth, 1983; Oades et al., 1986; Oades and Halliday, 1987). The DA neurons in the VTA, furthermore, are innervated by neurons of the MR, and have been shown by electron microscopy to receive synaptic contacts from 5-HT axon terminals (Herve et al., 1987; Oades and Halliday, 1987). Thus, a neurokinin---5-HT---DA circuit which mediates behavioral arousal can be postulated. In fact, we have unpublished data which shows that a low dose of the DA antagonist, haloperidol (200  $\mu\text{g}/\text{kg}$ , i.p.), can block the LMA effects of intra-MR DiMe-C7.

This system is distinct from the direct neurokinin-VTA projection which modulates LMA (Stinus et al., 1978; Kelley et al., 1979, 1985; Kalivas et al., 1985; Elliott and Iversen, 1986). Since the hyperactivity produced by intra-raphé infusions of neurokinins can be blocked by ibotenic acid lesions of the MR, the neurokinin-raphé induced increases LMA is not due to diffusion of the neurokinins to the adjacent VTA. In order to determine whether a MR-5-HT---VTA-DA link is critical for intra-MR neurokinin induced hyperactivity, it would be necessary to perform lesions of the VTA with the catecholamine neurotoxin, 6-hydroxydopamine (6OHDA). These studies are in progress.

Alternatively, it is possible that the intra-raphé neurokinin induced increase in LMA is mediated by a descending 5-HT projection.

The MR has been shown to send 5-HT as well as non-5-HT projections to the nucleus raphe magnus and obscurus in the rat, and the reticular formation and raphe pallidus in the cat (Brodal et al., 1960 a,b; Bobillier et al., 1975; Gallagher and Pert, 1978; Beitz, 1982). No direct spinal projections from the MR have been observed, although projections to cervical levels originating in the reticular formation, just lateral to the MR, have been reported (Bowker et al., 1981).

The MR 5,7-DHT lesions led to increases in amygdaloid DA and DOPAC levels and to increased DOPAC levels in the medial frontal cortex (Table III). No changes in DA or DOPAC levels were found in the nucleus accumbens or neostriatum. These observations suggest that damage to MR 5-HT neurons results in enhanced DA utilization in select forebrain regions and support the view that 5-HT fibers modulate the activity of mesencephalic DA neurons. The increases in DOPAC levels in the medial frontal cortex are in contrast to those observed following electrolytic lesions of the MR (Herve et al., 1981). This discrepancy, however, may be due to the non-specificity of electrolytic lesions as a technique to deplete 5-HT concentrations (Lorens, 1978). Although 5-HT neurons are known to innervate the basal ganglia, the MR 5,7-DHT lesions did not affect the DA and DOPAC levels in the neostriatum. This observation is in agreement with results obtained following electrolytic lesions (Dray, 1981; Soubrie et al., 1984). In fact, only electrolytic lesions of both the dorsal and median raphe nuclei have been observed to produce transient changes in striatal DOPAC levels (Juorio and Greenshaw, 1986).

The 5,7-DHT injections into the MFB, following pretreatment with the NE and DA reuptake inhibitor, nomifensine, produced falls in the DA, DOPAC, and NE levels in all the areas assayed, except the amygdala, suggesting incomplete protection of catecholamine neurons. The decreases in catecholamine parameters were between 20-40% and not nearly as great as those for 5-HT and 5-HIAA. The decreases in catecholamine levels, however, were not as extensive as that observed with MFB lesions with no pretreatment (Waddington and Crow, 1979). Baumgarten et al. (1981) have suggested that a combination of nomifensine and desmethylimipramine may provide optimal selectivity of 5,7-DHT for 5-HT neurons. However, it is important to point out that although the MFB lesions did produce 20-40% falls in forebrain catecholamine and metabolite levels, these lesions did not block the LMA effects of intraraphe infusions of the neurokinins.

The data from the present study indicate that raphe NK-2 and NK-3 receptor mediated hyperactivity depends upon 5-HT neurons. The results suggest that a 5-HT projection to the DA cell bodies of the ventral tegmental area may be important. Alternatively, a 5-HT projection to other brain stem sites cannot be ruled out. NK-2 mediated hyperactivity, on the other hand, may depend, in part, upon ascending 5-HT fibers.

### B. Anatomical Studies

The present series of experiments were undertaken in order to thoroughly examine the origin of the afferents to the MR with the eventual goal of determining the source of its neurokinin innervation.

The optimal method for accomplishing these goals is to use a retrogradely transported fluorescent dye, in combination with the indirect immunofluorescence method of Coons (1950, 1958; Bjorklund and Skagerberg, 1979; van der Kooy and Steinbusch, 1980; Skirboll and Hokfelt, 1983; Skirboll et al., 1984; Shiosaka and Tohyama, 1986). Fluorescent tracers have been shown to provide the most sensitive method for visualizing retrogradely labeled neurons (Skirboll et al., 1984). The fluorescent dye chosen to study the afferent projections to the MR was Fluoro-Gold (FG). This dye can be visualized following ultraviolet excitation, is intensely fluorescent, extremely resistant to fading, has a high signal-to-noise ratio, does not leak from cells, produces excellent dendritic filling, and can be readily combined with other fluorescent dyes and tracing methods (Schmued and Fallon, 1986; Wessendorf et al., 1987; Reep et al., 1988). Initially, we dissolved FG in double-distilled water and injected small (25-75 nl) volumes using 30 gauge tubing and a microsyringe pump. Despite the small volumes infused, this method resulted in extensive diffusion of the dye out of the MR and along the injection tubing. Therefore, the method of application adopted was to iontophorese the FG solution. This procedure produced very small, well demarcated injection sites (see Fig. 17f) with no diffusion along the micropipette track or into adjacent structures.

FG can be taken up and retrogradely transported by cut or damaged neuronal processes but not by intact fibers (Schmued and Fallon, 1986). Since fiber systems pass through and just lateral to the MR, it is possible that the FG may have been taken up by fibers damaged by the

micropipette. However, this type of non-specific uptake was probably very minimal because the concentration of the FG solution was low (2.0%) and the injection sites were very discrete (Schmued and Fallon, 1986). Nevertheless, the sources of the afferent projections to MR observed in the present study should be corroborated by employing an anterograde tracer, such as Phaseolus vulgaris leucoagglutinin (PHA-L).

Little attention has been focussed on the afferent inputs to the MR in the rat. In fact, only one systematic retrograde tracing study has been reported (Aghajanian and Wang, 1977). This was the first and only attempt we are aware of to document the source of the afferents to the midbrain raphe nuclei. However, this study qualitatively compared the afferents to the MR with those to the DR and pontine reticular formation. The present study differs from the work of Aghajanian and Wang (1977) in a number of respects. We employed a more sensitive retrograde marker (Schmued and Fallon, 1986; Shiosaka and Tohyama, 1986) and used a semi-quantitative approach to map the perikaryal origins of MR afferents in coronal sections obtained throughout the brain. The density of the various projections was inferred from the number of labeled cells found within a given structure. The data are diagrammed in Fig. 17 and are discussed below by region.

The regions found to contain to greatest numbers of retrogradely labeled cells included: the medial frontal and cingulate cortices, the septal area, the ventral pallidum/substantia innominata, the lateral habenular nucleus, the zona incerta, the lateral habenular nucleus, the lateral hypothalamus, the interpeduncular nucleus, the central gray, and the laterodorsal tegmental nucleus. Retrogradely labeled neurons

were conspicuously absent from a number of major forebrain structures, including: the caudate-putamen, the nucleus accumbens, the amygdaloid complex, the hippocampal formation, and entorhinal cortex.

#### Cortical Afferents

The only FG-labeled cells found in the cerebral cortex were located in the medial frontal cortex, and in the medial wall of the cingulate gyrus. Projections from these areas to the raphe have been described previously (Leonard, 1969; Aghajanian and Wang, 1977; Dr. E. J. Neafsey, personal communication, 1988). Pre-frontal cortical stimulation also has been reported to affect the firing rate of raphe neurons (Hardy and Haigler, 1985). Since an aspartatergic projection from the frontal cortex to the ventral tegmental area has been suggested (Christie et al., 1985), it is possible that the corticoraphe projection also may be aspartatergic. Although the MR has been shown to project extensively to other areas of the cerebral cortex, including the parietal and occipital regions, only the medial frontal and cingulate cortices were found to send fibers back to the MR (O'Hearn and Molliver, 1984; Parnavelas et al., 1985; Waterhouse et al., 1986; Kosofsky and Molliver, 1987; Molliver, 1987). Notably, the number of FG-labeled cells in these regions was very large (see Fig 17a-g). The possible function of cortical-raphe interactions will be discussed below (see pp. 179-180).

### Septal Afferents

FG filled cells were found in the dorsal and intermediate parts of the lateral septal area, in the medial septal nucleus, in the vertical and horizontal limbs of the diagonal band of Broca, and in the bed nucleus of the stria terminalis. These observations confirm previous reports of efferents from these structures to the MR as demonstrated by the retrograde transport of the fluorescent tracer true blue, and by the anterograde transport of PHA-L and [<sup>3</sup>H]leucine (Swanson and Cowan, 1979; Swanson, 1982; Tomimoto et al., 1987). In the present study, the largest number of labeled cells observed in the septal region were localized in the horizontal limb of the diagonal band, with minor contributions from the remaining septal nuclei. This observation appears to disagree with the results obtained by Aghajanian and Wang (1977). These authors reported a minor projection from the diagonal band to the MR, and an apparently insignificant projection from the bed nucleus.

### Basal Forebrain

The results indicate that afferents to the MR originate in the ventral pallidum, lateral preoptic area, and the rostral portion of the substantia innominata. Afferents to the MR from this portion of the rat forebrain have been observed by some but not all authors. For instance, Swanson et al. (1987) reported afferents to the MR from the medial preoptic area. However, their PHA-L injection included parts of the nucleus of the diagonal band and lateral pre-optic area which may have confounded their results (Swanson et al., 1987). An injection of PHA-L confined to the substantia innominata, as well as an injection

into the caudal portion of the lateral preoptic area resulted in labeled fibers and terminals in the region of the MR (Swanson et al., 1984). These observations have been confirmed by several other laboratories (Aghajanian and Wang, 1977; Tomimoto et al., 1987). A projection to the MR which originates in the ventral pallidal region also has been described by Haber et al. (1985) using the anterograde transport of tritiated amino acids. These authors also observed a few fibers along the outermost lateral border of the MR and adjacent pontine reticular formation following an injection of tritiated amino acids into the globus pallidus (dorsal pallidum; Haber et al., 1985). Retrogradely labelled neurons in these regions were not observed in the present study, perhaps due to the discrete FG infusions into the MR. The lack of retrogradely labelled neurons in the globus pallidus also has been noted by others (Aghajanian and Wang, 1977).

#### Habenular Complex

The results confirmed that a large number of cells in the lateral habenular nucleus project to the MR. These projections, which comprise a portion of the dorsal diencephalic conduction system (Sutherland, 1982), reach the midbrain via the fasciculus retroflexus (Akagi and Powell, 1968; Pasquier et al., 1976; Aghajanian and Wang, 1977; Herkenham and Nauta, 1979). These habenuloraphe fibers may pass through or around the interpeduncular nucleus (Akagi and Powell, 1968; Herkenham and Nauta, 1979). The principal target for medial habenular nucleus efferents is the interpeduncular nucleus (Herkenham and Nauta, 1979). Following an injection of tritiated amino acids into the medial

habenular nucleus, Herkenham and Nauta (1979) also found fibers and terminals within the MR. This observation is in disagreement with the present and other studies (Pasquier et al., 1976; Aghajanian and Wang, 1977). It should be noted, however, that in the present study, in all cases, one or two weakly labelled cells were found in the very dorso-lateral portion of the medial habenular nucleus, bordering on the lateral nucleus. Therefore, it is possible that a few neurons in the medial habenular nucleus project to the MR as suggested by Herkenham and Nauta (1979), but not to the extent to which they describe.

#### Dorsal Thalamus

The only dorsal thalamic region found to project to the MR was the ventromedial nucleus. Although earlier studies have reported a projection from the DR to the ventromedial thalamic nucleus (Herkenham, 1979), there are no reports of any connections between this nucleus and the MR. Shammah-Lagnado and colleagues (1987), however, observed afferents to the pontine reticular formation from a group of cells bordering the zona incerta and the ventromedial thalamic nucleus.

#### Zona Incerta

A significant projection from the zona incerta to the MR was identified. Although efferents from the midbrain raphe to the zona incerta have been described (Shammah-Lagnado et al., 1985), efferents from this region to the raphe have not been observed, except for a projection to the nucleus raphe magnus (Carlton et al., 1983).

### Hypothalamus

In the present study, FG-filled neurons were visualized in the following hypothalamic regions: the anterior hypothalamic area; the lateral nucleus; the paraventricular nucleus; the dorsomedial nucleus; the ventromedial nucleus; the posterior hypothalamic area; and, the mammillary complex. Aghajanian and Wang (1977) described afferents which originate in the anterior hypothalamic nuclei and terminate in the midbrain reticular formation but not in the MR. Hypothalamic afferents to the pontine reticular formation also have been reported by other investigators (Shammah-Lagnado et al., 1987). Saper and colleagues (1979) demonstrated lateral hypothalamic projections to the MR in the rat. Labeled fibers have been observed in the MR following the injection of tritiated amino acids into the dorsomedial hypothalamus and into the premammillary region (Krieger et al., 1979; Swanson et al., 1984). Tritiated amino acid injections into the ventromedial hypothalamus labeled fibers in the DR but not in the MR (Krieger et al., 1979). Projections from the paraventricular nucleus to the brain stem also have been reported (Armstrong et al., 1980; Conrad and Pfaff, 1976), but paraventricular connections have not.

FG-labeled perikarya were visualized throughout the mammillary complex. Following the injection of tritiated amino acids into the mammillary nuclei, Cruce (1977) observed labeled fibers in the lateral border of the MR, as well as in the ventral and dorsal nuclei of Gudden, but found no evidence for synaptic terminals. These mammillary projections appear to be reciprocated (Shibata, 1987).

### Substantia Nigra and Ventral Tegmental Area

A projection to the MR from the pars compacta of the substantia nigra, which contains the A9 DA cell group (Dahlstrom and Fuxe, 1964), was identified in the present study. Nigroraphe connections have been reported previously (Pasquier et al., 1977; Fallon and Loughlin, 1985). In comparison to its projection to the DR, however, the nigral projection to the MR appears to originate in only a few cells (see Fig. 17j,k).

In contrast to the substantia nigra, the ventral tegmental area, which contains the A10 DA cell group (Dahlstrom and Fuxe, 1964), was found to send a significant projection to the MR. Ventral tegmental DA and non-DA projections to the MR have been reported by others (as reviewed in Oades and Halliday, 1987).

The retrorubral field, which includes the A8 DA cell group (Dahlstrom and Fuxe, 1964), the rostral and caudal linear nuclei, and the interfascicular nucleus also are the source of afferents to the MR. These projections have been observed previously and most likely are DA and non-DAergic in nature (Fallon and Loughlin, 1985).

### Central Gray and Cuneiform Nucleus

Projections from the periaqueductal gray and adjacent cuneiform nucleus were noted in the present study. Although these regions have been reported to receive projections from the midbrain raphe, reciprocal connections have not been observed (Conrad et al., 1974; Andrezik and Beitz, 1985). However, both of these regions appear to project to the nucleus raphe magnus (Beitz, 1982; Carlton et al., 1983; Lakos and

Basbaum, 1988). Some of these fibers may contain neurotensin (Beitz, 1982). Since the midbrain raphe contains neurotensin fibers and binding sites (Kessler et al., 1987; Shipley et al., 1987), it is possible that afferents to the MR which originate in the central gray are neurotensinergic.

#### Interpeduncular Nucleus

The interpeduncular nucleus (IPN) is a major source of afferent fibers to the MR. Recently, Groenewegen and colleagues (1986) detailed the cytoarchitecture and fiber connections of the IPN. They reported that the MR is one of the major targets for IPN efferents. These raphe projections originate in distinct IPN subnuclei (Groenewegen et al., 1986). In the present study, retrogradely labeled neurons were found throughout the IPN. There also is some evidence that the projection from the IPN to the MR is excitatory (Maciewicz et al., 1981).

#### Gudden's Tegmental Nuclei

In the present study, numerous FG-labeled perikarya were found in the ventral tegmental nucleus of Gudden (VTG). This observation is in agreement with reports which describe reciprocal connections between the MR and the VTG (Morest, 1961; Petrovicky, 1973, 1985 a,b). Although the dorsal tegmental nucleus of Gudden (DTG) has been reported to project to the MR (Morest, 1961; Petrovicky, 1973), we failed to observe any retrogradely labeled neurons in the DTG. However, as described below, the adjacent laterodorsal tegmental nucleus does send a massive projection to the MR. The DTG and VTG, however, both project

to the adjacent pontine reticular formation (Petrovicky, 1985 a,b; Shammah-Lagnado et al., 1987).

#### Reticulotegmental Nucleus

Based on the number of labeled cells discerned, the pontine reticulotegmental nucleus (RTg) is the source of a minor projection to the MR (see Fig. 17m-o). The major target of RTg efferents is the cerebellum (Flumerfelt and Hryciyshyn, 1985). RTg projections to the MR have not been described previously.

#### Pedunculo pontine and Microcellular Tegmental Nuclei

The pedunculo pontine tegmental nucleus (PPTg) was found to send projections to the MR. The PPTg is located in the "mesencephalic locomotor region" (Skinner and Garcia-Rill, 1984; Garcia-Rill et al., 1987). Although it has been reported to project to other mesencephalic structures, such as the ventral tegmental area, the substantia nigra, the superior colliculus, and periaqueductal gray (Steeves and Jordan, 1984; Garcia Rill et al., 1987), PPTg projections to the MR have not been previously described. The PPTg contains cholinergic neurons which innervate the substantia nigra as well as the IPN (Armstrong et al., 1983; Woolf and Butcher, 1985, 1986; Paxinos and Butcher, 1985; Clarke et al., 1987; Beninato and Spencer, 1987; Jones and Beaudet, 1987; Rye et al., 1987). It thus is possible that the PPTg may be a source of a cholinergic projection to the MR.

The microcellular tegmental nucleus (Paxinos and Butcher, 1985), which lies dorsolateral to the PPTg, also was found to project to the

MR (see Fig. 17m,n). Although this nucleus is intensely stained by acetylcholinesterase histochemistry, and evidences SP-like immunoreactivity, relatively little is known about its connectivity or physiology (Paxinos and Butcher, 1985).

#### Laterodorsal Tegmental Nucleus

Another major source of afferent projections to the MR is the laterodorsal tegmental nucleus. This nucleus contains a large number of cholinergic neurons, and is the origin of an ascending cholinergic system, the dorsal tegmental pathway (Armstrong et al., 1983; Shute and Lewis, 1967). Using the PHA-L anterograde tracing technique, Satoh and Fibiger (1986) were the first to demonstrate a projection from this region to the MR. These authors suggest that this projection may be cholinergic because of the high levels of choline acetyltransferase activity in the MR (Kobayashi et al., 1975). However, it is also possible that this pathway may contain SP or one of the other neurokinins, since SP-like immunoreactive perikarya have been discerned in the laterodorsal tegmental nucleus (Sakanaka et al., 1982; Vincent et al., 1983).

#### Raphe Nuclei

MR afferents from the dorsal (DR) and pontine raphe nuclei, and from the nucleus raphe magnus and obscurus, were observed. A projection to the MR from the DR, but not from the other raphe nuclei, has been reported (Bobillier et al., 1975). These findings suggest that raphe-raphe connections may provide a means for 5-HT neurons in the MR to be regulated by 5-HT neurons in other raphe nuclei.

### Medial Nucleus of the Cerebellum

The medial cerebellar nucleus, the rat equivalent of the fastigial nucleus (Beitz and Chan-Palay, 1979 a,b), was found to project to the MR. The only previous report of a cerebellar input to the midbrain raphe was based on the degree of retrograde degeneration observed following lesions of the fastigial nucleus in the cat (Brodal et al., 1960b). In the present study, retrogradely labeled neurons in the medial nucleus were detected in all rats (see Fig. 17s and Fig. 18e). No other cerebellar regions were found to contain FG-filled cells.

### Visual System Afferents

Afferents to the MR were found to originate in the superior colliculus and anterior pretectal nucleus. Both of these regions are involved in the processing of visual information. It should be noted that MR afferents from the lateral geniculate body and occipital cortex were not observed. Although the midbrain raphe sends 5-HT and non-5-HT fibers to various components of the visual system, including the superior colliculus, lateral geniculate body, and retina (Beitz et al., 1986; Dolabela de Lima and Singer, 1987; Villar et al., 1987), these areas have not been reported to innervate the raphe (for a review, see Sefton and Dreher, 1985). However, fibers emanating from these areas descend just lateral to the MR in the tectospinal tract (Papez and Freeman, 1930; Waldron and Gwyn, 1969). It is possible that collateral branches from this system synapse upon cells within the raphe. Alternatively, it is possible that FG may have been taken up by fibers in

the tectospinal tract which may have been damaged by the glass micropipette. An anterograde tracing study utilizing PHA-L iontophoresed into the superior colliculus and pretectal nucleus would help clarify this issue.

### Trigeminal Nuclei

Two nuclei in the trigeminal complex were found to project to the MR: the principal sensory nucleus, and the motor nucleus of the trigeminal nerve. The trigeminal region may receive 5-HT and enkephalinergic projections from the midbrain raphe (Travers, 1985; Beitz et al., 1987), but trigemino-MR projections have not been described [see Travers (1985) and Tracey (1985) for reviews]. Ascending and decussating fibers emanating from the trigeminal complex pass immediately dorso-lateral to the MR. It thus is possible that some synapse upon cells in the raphe (Huerta et al., 1983; Smith, 1973). Alternatively, it is possible that these trigeminal cells were labeled because of uptake of FG by fibers damaged by the micropipette.

### Auditory Afferents

The present study localized afferents to the MR which originated in areas that make up part of the auditory system in the rat (Webster, 1985): the medial geniculate nucleus, inferior colliculus, and the ventral and lateral nuclei of the lateral lemniscus. Interconnections between these areas and the MR have not been reported previously.

### Vestibular Inputs

Three nuclei associated with the vestibular system in the rat (Mehler and Ruberstone, 1985) were found to project to the raphe: the lateral and medial vestibular nuclei, and the prepositus hypoglossal nucleus. Connections between the vestibular complex and the MR have not been previously described.

### Noradrenergic Afferents

Projections to the MR were observed to originate in areas containing norepinephrine (NE) cell groups: A4 and A6 of the locus ceruleus, and A5 and A7 of the lateral brain stem reticular formation (Dahlstrom and Fuxe, 1964). Previous reports have indicated that the MR receives its primary noradrenergic innervation from the lateral tegmental system which arises in the A1, A5 and A7 cell groups. The DR and MR appear to receive only a minor contribution from the locus ceruleus (A4 and A6; Levitt and Moore, 1979). WGA-HRP injections into the region of the A5-NE containing cells did not result in labeled fibers in the DR or MR (Byrum and Guyenet, 1987). However, dopamine- $\beta$ -hydroxylase immunocytochemistry has confirmed the presence of noradrenergic fibers and terminals in the mesencephalic raphe (Swanson and Hartman, 1975). Regions adjacent to the MR, including the dorsal tegmental nucleus of Gudden, the VTA, and the pontine reticular formation receive afferents from the LC (Levitt and Moore, 1979; Shammah and Lagnado et al., 1987).

### Reticular Formation

A number of reticular structures were found to send efferent projections to the MR including the deep mesencephalic nucleus, the oral and caudal parts of the pontine reticular formation, and the gigantocellular and lateral gigantocellular reticular nuclei. These regions have been shown to receive projections from the MR (see review by Steinbusch and Niewenhuys, 1983), but have not been previously reported to send efferents to the MR. Interconnections between the reticular formation and the raphe are not surprising in light of the similarity of their afferent and efferent connections (Andrezik and Beitz, 1985; Shammah-Lagnado et al., 1985). In fact, in their original description, Taber et al. (1960) considered the raphe nuclei as forming the medial extension of the reticular formation.

### C. Immunocytochemical Findings

#### Localization of Neurokinin-like Immunoreactivity in the

#### Midbrain Raphe and Surrounding Regions

The first step in determining the neurokinin innervation of the MR was to characterize the SP-like (SPLI) and neurokinin-like (NKLI) immunoreactivity. This was accomplished utilizing antibodies directed against SP and NKA/NKB (antisera SP2 and NKA2; Brodin et al., 1986), as well as anti-SP antisera obtained commercially (< 10% cross-reactivity with NKA and NKB; Dr. Mary Frick, Incstar, Inc, personal communication, 1988). These antibodies produced SPLI and NKLI staining which was similar in distribution (see Fig. 19). These observations are consis-

tent with those of other investigators who found SPLI in the MR and the trigeminal complex (Ljungdahl et al., 1978 a; Cuello and Kanazawa, 1978; Shults et al., 1984). Because of the cross-reactivity of the NKA/NKB antisera employed, and because of the similar distribution and probable co-localization of NKA and SP in the CNS, the immunoreactive methods used were unable to distinguish between SPLI and NKLI.

In order to confirm the presence of the neurokinins in the mid-brain raphe, a collaborative study was undertaken with Dr. Ernst Brodin (Karolinska Institute, Stockholm). In brief, adult male Sprague-Dawley rats were sacrificed, and tissue punches (1.5 mm in diameter and 1.0 mm thick; average weight = 3.4 mg/punch) were obtained from the MR, DR, IPN and rostral central gray. The tissue was frozen on dry ice, pooled into three samples for each area (one sample = 4-5 punches), then stored at  $-40^{\circ}\text{C}$  until analyzed by reverse-phase HPLC and radioimmunoassay (Brodin et al., 1986). The samples were assayed for SP with antisera SP2 (cross-reactivity with NKA/NKB and NPK, negligible) and for NKA/NKB with antisera NKA5 and E7 (which cross react with NKB, 90 and 240%, respectively; and, with NPK, 54 and 93%, respectively; Brodin et al., 1986; Lindefors et al., 1985 b; Dr. E. Brodin, personal communication, 1988). The SP-like and NKA-like concentrations, as well as the NKA/NKB and NKA/NPK ratios in each brain area are presented in Table IV.

Table IV: HPLC-RIA ANALYSIS OF MIDBRAIN TISSUE PUNCHES

<u>Region</u>	<u>SPLI</u>	<u>NKALI</u>		<u>NKA</u>	<u>NKA</u>
	Antisera <u>SP2</u> (fmol/sample)	<u>NKA5</u> (fmol/sample)	<u>E7</u> (fmol/sample)	NKB	NPK
MR	1971 ± 324	2679 ± 388)	2337 ± 331)	4.3	9.0
DR	1904 ± 418	2503 ± 567	2666 ± 413	4.4	8.2
CG	5630 ± 2430	3784 ± 572	4350 ± 922	5.4	6.4
IPN	2518 ± 1591	3526 ± 809	4343 ± 1044	1.9	5.0

Data represent mean ± SEM for three samples. Each sample represents punches from 4-5 animals. Cross-reactivities of the antisera were accounted for in the calculation of the ratios.

These data confirm that each of the four brain regions contain SP, NKA, NKB, as well as small amounts of NPK. It would appear that the three neurokinins exist within the raphe in roughly equal concentrations. These results, furthermore, underscore the need for utilizing biochemical methods in order to substantiate immunocytochemical findings.

#### Problems Associated With Peptide Immunocytochemistry

One of the objectives of the dissertation project was to determine the origin of the neurokinin innervation of the MR. To accomplish this goal, retrograde tracing using the fluorescent dye, Fluoro-Gold (FG), was combined with immunocytochemical techniques to localize the neurokinins. This method is recognized as a sensitive and useful

procedure for visualization of neurotransmitter-specific afferents to a particular brain region (van der Kooy and Steinbusch, 1980; Hokfelt et al., 1983; Skirboll and Hokfelt, 1983; Skirboll et al., 1984). The first step in this procedure is to identify perikarya which accumulate the dye by retrograde transport. The second step involves the visualization of neurons which contain the neurotransmitter. The final step involves the successful combination of the two.

Visualization of a particular transmitter can be accomplished by immunocytochemistry (Coons, 1950, 1958; Sternberger, 1979; Shiosaka and Tohyama, 1986). The immunocytochemical demonstration of a substance, however, is associated with a number of problems. These include: the sensitivity and specificity of the primary antisera; modification or loss of antigenic binding sites due to fixation or sectioning; lack of antibody penetration; choice of buffers; steric hindrance preventing antibody binding; and the so-called "Bigbee" effect (Bigbee et al., 1977), in which high concentrations of antigen and primary antibody prevent the binding of the secondary antibody (Larsson, 1983; Sofreniew, 1983; Van Leeuwen, 1986). All of these factors can be the source of false-negative/positive results, and must be eliminated during the development of a staining procedure for a given substance.

Peptide immunocytochemistry, furthermore, involves some unique obstacles which must be overcome. Since neuropeptides are synthesized from large precursor molecules which originate in the soma and are transported and processed enroute to the nerve terminal, the concentration of the peptide in the cell body is typically very low (Hokfelt et al., 1980). In order to visualize peptide-containing perikarya,

animals usually must be pretreated with colchicine so that the peptide accumulates within the soma (Dahlstrom, 1971; Cuello et al., 1983; Larsson, 1983). The charge of the peptide during the processing steps, determined by the pH of the buffer system employed, also is critical to the success of the staining procedure (Van Leeuwen, 1986).

Another problem associated with peptide immunocytochemistry is that the antibodies employed may cross-react with the precursor or similar molecules which contain the antigenic site against which the antibodies are directed (Larsson, 1983; Hokfelt et al., 1980; Block and Hoffman, 1987). Thus, the antibodies may enable the visualization of a particular group of immunoreactive cells, but subsequent studies utilizing more specific antisera may give conflicting results. This has been the case for somatostatin as well as the opioid neuropeptides (Hokfelt et al., 1980). Because of the similarity in the structure of members of a related group of peptides, cross-reactivity can be a significant problem. In fact, the high cross-reactivity of the antibodies utilized in some of the first SPLI mapping studies has precluded a definitive localization of SP-specific neurons and processes (Dr. Tomas Hokfelt, personal communication, 1986). Another problem associated with peptide immunocytochemistry is that antisera directed against a particular antigenic site may not always be recognized in tissue sections because of the three-dimensional structure of the peptide in-vivo (Larsson, 1983), or because of its membrane binding properties. This particular problem is not typically encountered with other techniques, such as radioimmunoassay or HPLC (Brodin et al., 1986; Larsson, 1983). Since these procedures are performed on peptides extracted from tissue,

more of the molecule is accessible to the antibody, thus allowing a better opportunity for binding, and increased specificity for antisera directed against the unique portions of the peptide chain (Larsson, 1983; Brodin et al., 1986).

#### Neurokinin Immunoreactivity in Dorsal Root Ganglia

In order to control for the ability of our procedures to localize SPLI and NKLI cell bodies, SPLI and NKLI were first demonstrated in spinal dorsal root ganglia (DRG), a tissue known to be rich in neurokinin cell bodies (Hokfelt et al., 1975; Dalsgaard et al., 1982, 1985; Fuxe et al., 1983; Leach et al 1985; Gibbins et al., 1987). Sections through the DRG from both intrathecally colchicine-treated and untreated rats were processed as described in Chapter III, Methods. Using this procedure, we were able to visualize both SPLI and NKLI cell bodies and fibers in the DRG. Although intrathecal administration of colchicine was used to arrest axonal transport and increase the cellular concentration of the peptides (Dahlstrom, 1971; Cuello et al., 1983; Dalsgaard et al., 1985), contrary to our expectations, more perikarya were stained in the untreated rat (Fig. 20), whereas better fiber staining was seen in the colchicine-treated rat. It is possible that the accumulation of material in the soma following colchicine treatment causes swelling and distention which hinders the binding of the antibody to the peptides. This physical disruption also could lead to better antibody penetration of individual fibers and hence better axonal staining.

Localization of Neurokinin Cell Bodies in the CNS

The mapping of SP-like immunoreactive cell bodies in the adult rat CNS was first reported in the seminal studies of Ljungdahl et al. (1978 a,b) and Cuello and Kanazawa (1978) and subsequently by Shults et al. (1984). Their findings, as well as those obtained in the present study, are summarized in Table V. As can be seen, the results overall are in general agreement.

Although neurokinin-like cell body staining was obtained in sections through the dorsal root ganglia (see Fig. 20), CNS perikaryal staining following colchicine pretreatment was not observed consistently in every brain region examined. In general, the best cell body staining was obtained in the brain stem, particularly in the central gray, IPN, DR and laterodorsal tegmental nucleus. With the exception of the IPN, this might be explained by the proximity of these regions to the ventricular system and, thus, their accessibility to the colchicine administered into the lateral ventricles (Ljungdahl et al., 1978 a). SPLI cell bodies in the IPN can be easily visualized in untreated rats (Cuello and Kanazawa, 1978; Ljungdahl et al., 1978 a; Artymyshyn and Murray, 1985; Contestable et al., 1987).

Table V: SUBSTANCE P-LIKE IMMUNOREACTIVE CELL BODIES IN THE CNS

<u>Region</u>	<u>Ljungdahl</u> <sup>1</sup>	<u>Shults</u> <sup>2</sup>	<u>Cuello</u> <sup>3</sup>	<u>Present</u> <sup>4</sup>
<u>Rhinencephalon</u>				
olfactory tubercle	+			
<u>Telencephalon</u>				
nucleus accumbens	++	++		
<u>septal nuclei</u>				
medial	+++	+++		+
lateral	++++	++++		+
diagonal band of Broca	+++	++		
caudate-putamen	+++	+	+	+
medial forebrain bundle area <sup>5</sup>	++			
interstitial nucleus of the anterior commissure	++++	++		
globus pallidus			+	
bed nucleus of the stria terminalis	++++	++++		
amygdala	+++	++		
preoptic area, medial	++++	+++		
<u>hypothalamus</u>				
suprachiasmatic nucleus	++	+		
anterior hypothalamic nucleus	+	+++		
paraventricular nucleus	+	+++		+
ventromedial nucleus	++++	+++		
dorsomedial nucleus	+++	++++		
lateral hypothalamic	++++	++++		
preammillary nucleus	++++			
posterior hypothalamic nucleus	+++			
<u>Diencephalon</u>				
subthalamic nucleus	+			
medial habenular nucleus	++++	++++	+	+++
zona incerta	++			
<u>Mesencephalon</u>				
central gray	++++	++++		+++
nucleus of Darkschewitsch	+++			
interpeduncular nucleus	++++	++++	++	++++
mesencephalic reticular formation	++			
superior colliculus	+++	+		
laterodorsal tegmental nucleus	+++	+++		+++
dorsal raphe	++	+		+
pontine reticular formation, caudal	+	+		

Table V (cont')

Mesencephalon (cont')

cuneiform nucleus	+++
dorsal parabrachial nucleus <sup>6</sup>	++
raphe pontis	++

Myelencephalon

raphe magnus	++++	++++	+++
raphe obscurus	+++	+	
raphe pallidus	+++	+	
medullary reticular formation	++++	++++	+
spinal vestibular nucleus	+	+	
nucleus of the solitary tract	+	+	
spinal trigeminal nucleus	+	+	
caudal trigeminal nucleus		+	
lateral cuneate nucleus	+		

1 Adapted from Ljungdahl et al. (1978): + = 1; ++ = 2-5; +++ = 6-20; ++++ = > 20 SP-immunoreactive cell bodies.

2 Adapted from Shults et al. (1984): + = "low"; ++ = "moderate"; +++ = "high"; ++++ = "highest" number of SP-immunoreactive cells bodies.

3 Adapted from Cuello and Kanazawa (1978). Since these authors were primarily interested in immunoreactive fibers and terminals, they did not use colchicine pretreatment and did not attempt to quantify the number of immunoreactive cell bodies encountered.

4 Present study: the rating scheme of Shults et al. (1984) was used.

5 The "magnocellular preoptic nucleus" of Paxinos and Watson (1986).

6 The "lateral parabrachial nucleus" of Paxinos and Watson (1986).

In the present study, SPLI cell bodies were observed in the septum, diagonal band of Broca, hypothalamus, and medial habenular nucleus, but not in every rat. Ljungdahl et al. (1978 a) also were unable to discern SPLI perikarya in a given area in every rat studied. They reported, furthermore, that intracerebral infusions of colchicine were necessary in order to visualize SPLI perikarya in certain areas (Ljungdahl et al., 1978 a). In addition, SPLI cell bodies were more easily seen and in some cases, exclusively, in young (1-3 week old) rats (Ljungdahl et al., 1978 a). These observations emphasize the importance of using optimal conditions for each brain region under study.

The problems (reviewed above pp. 156-159) encountered with neurokinin staining probably explain why most immunocytochemical studies of this family of peptides have focussed upon individual brain regions. Table VI provides a list of reports in which neurokinin-like cell bodies have been visualized in the CNS.

The lack of perikaryal neurokinin-like staining in certain CNS regions could be due to the low concentration of the peptide in the soma, even after colchicine pretreatment. This may be especially true for neurons with long and/or highly collateralized axons. An example is the striatonigral neurokinin system. This is probably the most extensively studied neurokinin system in the CNS (Jessel et al., 1978; Kanazawa et al., 1980; Bolam et al., 1983, 1986; Gerfen, 1985; Ritter, 1985; Arai et al., 1987; Bannon et al., 1987; Chesselet et al., 1987; Kawai et al., 1987; Li et al., 1987; Nylander and Terenius, 1987; Sivam et al., 1987; Voorn et al., 1987; Diez-Guerra et al., 1988).

Table VI: SUBSTANCE P-LIKE IMMUNOREACTIVITY IN SELECTED AREAS

<u>Region</u> <sup>1</sup>	<u>Visualization Method</u> <sup>2</sup>	<u>Source</u>
nucleus accumbens	PAP	Pickel et al., 1988
caudate (cat and ferret)	PAP	Izzo et al., 1987
(cat)	PAP	Beckstead, 1987
	PAP	Penny et al., 1986
	PAP	Bolam et al., 1983
arcuate nucleus & median eminence	PAP and colloidal gold	Tsuruo et al., 1987
tuberomammillary nucleus	FITC and PAP	Kohler et al., 1985
medial habenular & interpeduncular nuclei	PAP	Contestabile, et al., 1987
hippocampus	PAP (SP and NKA)	Shults et al., 1987
	FITC and PAP	Davies and Kohler, 1985
(human)	FITC	Del Fiacco et al., 1987
dorsal raphe	PAP	Magoul et al., 1986
interpeduncular nucleus	PAP	Groenewegen et al., 1986
	PAP	Vu and Hamill, 1988
	PAP	Barr et al., 1987
	PAP	Artymyshyn and Murray, 1985
	FITC	Huitinga et al., 1985

Table VI (cont')

parabrachial nuclei	PAP	Block and Hoffman, 1987
nucleus of the solitary tract (monkey)	FITC PAP	Thor and Helke, 1987 Maley et al., 1987
medullary raphe & reticular formation	PAP PAP	Menetry and Basbaum, 1987 Hancock, 1984
brain stem (human)	PAP	Nomura et al., 1987
ventrolateral medulla (cat)	FITC PAP	Pilowsky et al., 1986 Ciriello et al., 1988

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<sup>1</sup> Species used is the rat, unless otherwise noted.

<sup>2</sup> Method of visualization: PAP = peroxidase antiperoxidase (light microscopy); FITC = fluorescein isothiocyanate (fluorescence microscopy)

Some investigators have had difficulty visualizing SP-like immunoreactive cell bodies in caudate nucleus (Ljungdahl et al., 1978 a; Bolam et al., 1983), while others presumably have had no difficulty, even without colchicine pretreatment (Cuello and Kanazawa, 1978). In contrast to the caudate nucleus, the IPN, with its relatively shorter projections and high concentrations of SP, is a structure in which SPLI cell bodies are readily stained under a variety of conditions.

Alternatively, it is possible that there is differential processing and expression of neurokinins in distinct pathways. The density and distribution of SP binding sites, cell bodies and fibers fluctuates during the pre- and post-natal period (Inagaki et al., 1982; Sakanaka et al., 1982; Quirion and Dam, 1986). This could explain why cell bodies in some brain regions are only visualized in very young (1-3 week old) rats (Ljungdahl et al., 1978 a).

It is worth noting that a number of recent studies have utilized the PAP or avidin-biotin-PAP technique for visualizing neurokinin cell bodies. These methods do not require specialized fluorescence microscopy and may be more sensitive because of the low concentrations of primary antibody which can be used (Hsu et al., 1981; Larsson, 1983; Vandersande, 1983; Van Leeuwen, 1986). A fluorescence immunocytochemical technique was used in the present study because of the sensitivity of the retrogradely transported fluorescent dyes which are available (Hokfelt et al., 1983; van der Kooy and Steinbusch, 1980; Skirboll and Hokfelt., 1983; Skirboll et al., 1984). Although other neuronal tracers, such as horseradish-peroxidase (HRP) and wheat-germ agglutinin (WGA), are available (Schwab et al., 1978; Lechan et al., 1981; Mesu-

lam, 1982), these suffer from the disadvantages of being taken up by fibers of passage and producing anterograde as well as retrograde labeling (Lechan et al., 1982; reviewed by Hokfelt et al., 1983). Improvements in specificity have come through conjugation of WGA with HRP and gold complexes, and their careful iontophoretic application into the brain (Gonatas et al., 1979; Baker and Spencer, 1980; Basbaum and Mentery, 1987). A disadvantage, however, is that these techniques require a special development step not needed with the fluorescent tracers (Hokfelt et al., 1983).

#### Combined MR Fluoro-Gold Injections with Neurokinin Immunocytochemistry

Neurokinin-like staining of selected brain regions was attempted using two rats which had received FG injections into the MR 10 days earlier. Since our best SPLI and NKLI staining was observed in brain stem areas, these regions were concentrated on for the combined retrograde tracing and immunocytochemical studies. Both FG and FITC labeled neurons were discerned in several brain structures, but especially in the rostral central gray, the DR, the IPN, and the laterodorsal tegmental nucleus. Double-labeled cells, however, were not observed. In one case, there may have been one or two double-labeled neurons in the laterodorsal tegmental nucleus, but on re-examination, the fluorophore appeared to have faded. Fig. 22e,f shows FITC and FG but not double-labeled cells in the same DR-section. Therefore, the neurokinin innervation of the MR probably does not originate in these brain regions.

Future Considerations for Determining the Neurokinin Afferents to the MR

Because of the inability to observe FG-labeling in neurokinin-like perikarya, the origin of the neurokinin innervation of the MR was not demonstrated. Thus, alternative approaches should be adopted. One approach would be to concentrate on the brain regions which have been shown to send fibers to the MR. Neurokinin-like staining then could be optimized for those regions and eventually combined with retrograde labeling. For instance, it might be necessary to utilize intracerebral injections of colchicine in order to bring out neurokinin-like perikarya staining (Ljungdahl et al., 1978 a; Bolam et al., 1983; Groenewegen et al., 1986). Alternatively, as suggested in recent articles (see Table VI), it may be necessary to utilize the PAP or avidin-biotin method of Hsu and colleagues (Hsu et al., 1981; Hsu and Reine, 1981), rather than fluorescent methods.

Another approach would be to place lesions in putative neurokinin-labeled nuclei or pathways which may innervate the MR, then analyze tissue punches of the raphe nuclei for neurokinin immunoreactivity. This method has been used extensively to study the striatonigral neurokinin system (Jessel et al., 1978; Kanazawa et al., 1980; Arai et al., 1987; Sivam et al., 1987; Voorn et al., 1987). A biochemical approach using HPLC coupled with RIA has been used successfully to determine the amount of neurokinin immunoreactivity in the MR and various other brain regions (see Table IV; Brodin et al., 1986; Lindfors et al., 1985). This approach has the advantage of being able to produce quantitative data concerning the neurokinin input to the

raphe and is being used in our collaborative study with Dr. Brodin's laboratory.

In situ hybridization histochemistry has been recently employed to study neurokinin distribution in the CNS (Chesselet et al., 1987; Warden and Young, 1988). This technique uses  $^{35}\text{S}$ -labeled probes specific for neurokinin mRNA transcripts. Although the probes are highly sensitive and avoid many of the problems encountered with immunocytochemistry, at present the technique appears to suffer from a lack of resolution. For example, Warden's and Young's (1988) recent report, which compares the distribution of the mRNAs for SP and NKB in the rat CNS, localizes SP transcripts in regions not previously shown to contain SP-like cell bodies. This would suggest that either there is a technical problem with their methods, or that some neurons in the CNS are inefficient with respect to gene transcription. The authors did not try to reconcile these discrepancies. Although these techniques offer great potential for localizing neuronal neurokinin processes, Warden's and Young's results must be interpreted carefully.

#### D. Function of Neurokinin-Induced Hyperkinesis

The results from the present study raise some important questions. What is the functional relevance of the behavioral arousal produced by neurokinin infusions into the MR? Is the animal exploring, searching for food, seeking a means of escape, or simply locomoting? What anatomical circuits mediate intra-MR neurokinin induced activation? The following discussion will address these questions.

## What is the Function of Intra-MR Induced Hyperkinesia?

### Open Field Studies

Measurement of LMA in enclosed photocell chambers does not permit an analysis of the animal's behavior. The animal could generate activity counts (photobeam breaks) by rotating, grooming, rearing, or biting (Dourish, 1987). Likewise, drug induced increases in activity could be manifested in several ways. For example, infusions of tachykinins into the ventral tegmental area induce increases in LMA as measured in photocell chambers, but when observed in an open field, the rat may be engaging in grooming, rearing or scratching (Elliott and Iversen, 1986). Peripheral and intracisternal injections of senktide, furthermore, have been reported to produce stereotypic movements in rats (Stoessl et al., 1987 a,b). Thus, we observed rats in an open field following neurokinin infusions into the MR.

It is generally accepted that exposure of an animal to a novel environment elicits both fear (neophobia) and curiosity. Immediately after placement in the center of the open field, a normal rat will move cautiously but directly to the wall and "freeze," defecate and urinate. The animal then will ambulate along the wall of the compartment seeking a means to escape. As the animal's fear attenuates, it begins to explore the apparatus. Eventually, the animal habituates to the chamber, and tends to sit or lie quietly near the wall, occasionally sauntering to another part of the arena. A number of factors affect an animal's response to a novel open field. These include the intensity and type of illumination and background noise; the degree of food

and/or water deprivation, the strain and sex of the rat; time of day and year; handling and prior experience; the presentation of reinforcing stimuli, such as electric shock, highly palatable foods, or small furry objects; and, how the animals are normally housed (Gray, 1972, 1982; Frankova et al., 1987). Although not well understood, pheromones also may play a role, especially when rats are tested sequentially in the same chamber. The open field used in the present study (see Methods, Chapter III) was designed to provide a mildly fearful environment with holes in the floor which could elicit exploratory responses. In addition, the animals were handled frequently and were well adapted to the injection procedure.

The results of the first two open field experiments showed that vehicle treated rats exhibited high levels of activity, and that much of their time was spent exploring the chamber. This was evidenced by the high incidence of rearing, head pokes, and center squares entered (see Fig. 10 and 11). Intra-MR infusions of DiMe-C7 and NKA did not lead to further increases in activity. In contrast, senktide, as well as muscimol (unpublished observations), produced increases in LMA.

In order to control for the effects of novelty on the behavioral arousal produced by neurokinin injections into the MR, the rats in the third open field experiment were adapted to the chamber prior to drug-testing. Not surprisingly, the vehicle treated rats exhibited a much lower level of activity than similarly treated rats exposed to the novel chamber (compare Fig. 12 and Fig. 14). MR infusions of both NKA and senktide produced increases in activity in rats which had been adapted to the open field arena. The increases in activity were mani-

fested predominantly as increases in wall squares entered during the first 15 minutes in the chamber. However, it is interesting to note that although both NKA and senktide produced significant increases in activity, NKA tended to increase exploratory behaviors: rears, head pokes, and center squares entered. Anxiolytic compounds produce similar effects (Gray, 1982). In contrast, senktide induced locomotion primarily along the periphery of the chamber. These observations suggest that the binding of senktide to an NK-3 receptor in the MR results in increased locomotion, whereas binding of NKA to an NK-2 receptor leads to enhanced exploration. Thus, activation of NK-2 receptors on 5-HT neurons in the MR may produce a mild anxiolytic effect.

#### Punished Drinking

The potential "anxiolytic" effects of intra-MR infusions of senktide were examined using a punished drinking paradigm (unpublished observations). Anti-anxiety drugs increase the rate of punished responding (File, 1987; Treit, 1985). Senktide (0-30 pmol) did not affect punished or unpunished drinking. Thus, activation of a MR NK-3 receptor does not appear to produce an anxiolytic effect. We are planning to examine the effects of NKA and the NK-1 agonist, septide, using this paradigm.

#### Effects on Food and Water Intake

To further investigate the functional significance of neurokinin induced LMA, we observed the effects of the peptides on food and water

intake. MR infusions of muscimol and the 5-HT<sub>1A</sub> agonist, 8-OHDPAT, have been demonstrated to increase locomotion and to induce increases in food and water intake in non-deprived rats (Dourish et al., 1985 a,b,c, 1986; Klitenick and Wirtshafter, 1986, 1987). Since the neurokinins also increase LMA, it is possible that they could stimulate ingestive behaviors as well. Studies conducted in our laboratory, however, have revealed that senktide infusions into the MR or DR produce decreases in both food and water consumption in non-deprived rats. No effects were observed in deprived animals. These effects of senktide on food intake are novel. Infusion of pmol doses of tachykinins into the lateral ventricles has been reported to produce an antidipsogenic effect in rats (Massi et al., 1986; De Caro et al., 1987). Thus, it is possible that the tachykinins may regulate water intake via the MR. It is interesting in this regard that Lorens et al. (1971) found that electrolytic midbrain raphe lesions produced transient increases in water consumption.

#### Effects on Reinforcement Processes and Pituitary-Adrenal Axis

Preliminary studies in our laboratory, using the conditioned place preference paradigm, suggest that intra-MR infusions of muscimol induce aversive effects, whereas DiMe-C7 injections may produce rewarding effects (Lee, J. et al., 1987). Infusions of muscimol into the MR, furthermore, were found to produce dose-dependent increases in the plasma levels of ACTH and corticosterone. These pituitary-adrenal hormones are secreted as a result of an animal's exposure to aversive or stressful stimuli (Bassett et al., 1973; File and Peet, 1980; Natel-

son et al., 1987). In contrast, MR infusions of DiMe-C7 do not alter the plasma levels of these hormones.

#### Neurochemical Effects

Muscimol has been shown to produce several behavioral effects when infused into the midbrain raphe, including hyperkinesia, hyperphagia, and hyperdipsia, as well as endocrine and electrophysiological changes. It has been reported that raphe-GABAergic neurons modulate forebrain 5-HT and DA turnover (Forchetti and Meek, 1981; Nishikawa and Scatton, 1985 a,b; Nishikawa et al., 1986).

The effects of intra-MR infusions of the neurokinins on 5-HT turnover have not been thoroughly examined. One study has reported that intra-MR administration of high doses of SP (4.0 and 10  $\mu$ g) produces an increase in 5-HT turnover in the hippocampus, measured as an increase in 5-HIAA concentration (Forchetti and Meek, 1982). This study, as well as preliminary evidence obtained in our laboratory using DiMe-C7, supports the view that the neurokinins modulate the activity of 5-HT neurons.

#### Neuroanatomical Considerations

Locomotion is composed of a complex series of neuronal events which involve spinal and brain stem signals which are monitored by cortical and diencephalic messages. Spinal feedback, as well as sensory input, can modulate any component of the circuit. Although the basic locomotor pattern generator involves a hard-wired spinal circuit, locomotion is dependent upon supraspinal influences (Carew, 1985; Arm-

strong, 1986). Some of the major supraspinal messages come from the mesencephalic and subthalamic locomotor regions, the nigrostriatal and mesoaccumbens DA systems, the pontomedullary locomotor sites, the red nucleus and rubrospinal tract, and the cerebellum (Armstrong, 1986). To implicate the MR in a complex series of neuronal connections, such as those involved in locomotion, requires anatomical documentation. The anatomical findings obtained in the present study will be discussed in relation to known locomotor circuits.

#### Mesencephalic Locomotor Region

A "mesencephalic locomotor region" has been localized in the posterior midbrain. Electrical stimulation of this region induces locomotion in cerveau isole cats (Grillner and Shik, 1973). The anatomy and physiology of this region had been studied extensively by Garcia-Rill and colleagues (Garcia-Rill et al., 1983 a,b; Garcia-Rill, 1983; Garcia-Rill and Skinner, 1987 a,b). An homologous region, primarily involving the pedunculopontine nucleus (PPTg), has been demonstrated in the rat (Skinner and Garcia-Rill, 1984; Garcia-Rill et al., 1987). GABA neurons appear to play an important role in the modulation of locomotor movements by this area (Imperato and DiChiara, 1981; Childs and Gale, 1983; Garcia-Rill et al., 1985). The mesencephalic locomotor area, furthermore, is reciprocally connected with the nucleus accumbens, entopeduncular nucleus, and motor cortex (Garcia-Rill et al., 1981, 1983, 1985; Saper and Lowey, 1982; Brudzynski and Mogenson, 1985; Brudzynski et al., 1986; Yang and Mogenson, 1987; Swerdlow and Koob, 1987; Scarnati et al., 1987). The mesencephalic locomotor area

and basal ganglia also appear to be functionally interconnected (Garcia-Rill, 1986).

There have been no reports, to our knowledge, concerning the relationship between the MR and the PPTg in the rat. The present anatomical study represents the first observation of a direct link between the PPTg and the MR. Previous studies have indicated that a number of cells in the PPTg contain choline acetyltransferase (Sofroniew, 1985 a,b) and NADPH-diphorase (a marker reputedly specific for cholinergic neurons in the PPTg; Goldsmith and van der Kooy, 1988), suggesting that the PPTg may be the source of a cholinergic projection to the MR. Additional electrophysiological and lesion experiments are needed to verify the functional relationship between these brain stem nuclei.

#### Subthalamic Locomotor Region Connections

Another region which has been implicated in locomotion is the "subthalamic locomotor area" (Armstrong, 1986). This diencephalic area comprises the fields of Forel, the subthalamic nucleus, zona incerta, lateral hypothalamus, and the mammillary nuclei (see Armstrong, 1986). These structures have been shown anatomically and physiologically to project to and modulate neuronal activity within the PPTg (Swanson et al., 1984, 1987; Mogenson et al., 1985; Mogenson and Wu, 1986). As discussed previously (p. 143), several components of the subthalamic locomotor area have reciprocal connections with the MR, including the zona incerta, the lateral hypothalamus, and the mammillary nuclei.

Therefore, connectivity with the subthalamic locomotor area provides another means for MR neurons to communicate with motor circuits.

#### Lateral Hypothalamic Locomotor Connections

Electrical stimulation of the lateral hypothalamus has been shown to affect locomotion (Gross and Sinnamon, 1986; Sinnamon and Stopford, 1987; Sinnamon et al., 1987). The relationships between this region and the ventromedial midbrain have been examined (Sinnamon et al., 1987; Sinnamon and Stopford, 1987). It would appear that the MR and adjacent structures may be involved in the locomotion induced by electrical stimulation of the lateral hypothalamus. Lesions of the dorsal midbrain, including the PPTg, did not affect electrically stimulated LMA (Sinnamon and Stopford, 1987). Sinnamon and colleagues (1987) present evidence that electrical stimulation of the midbrain raphe can produce a variety of locomotor effects, ranging from facilitation to inhibition when observed in the presence or absence of concurrent hypothalamically-induced locomotion (Sinnamon et al., 1987). Two caveats apply to this type of research: 1) the effects were observed in anesthetized preparations and thus may not necessarily reflect locomotion in awake animals; and, 2) electrical stimulation produces both antidromic and orthodromic activation of fibers of passage, as well as stimulation of interstitial cell bodies. Thus, interpretation of these experiments can be quite difficult.

### Association with the Mesotelencephalic DA Systems

The mesotelencephalic DA system is composed of the mesostriatal and mesolimbocortical DA systems, originating in the A8-10 DA cell groups (Bjorklund and Lindvall, 1984). A vast amount of literature has accumulated in recent years implicating the mesencephalic DA cell groups and their terminal regions in locomotion. A number of investigators also have implicated MR-5-HT neurons in the regulation of these pathways (Chase, 1974; Jacobs, 1974; Dray et al 1976; Hery et al., 1980; Lee and Geyer, 1984 a,b; DiSimoni et al., 1987; Herve et al, 1987; Lee, E.H.Y. et al., 1987; Mori et al., 1987). The mesencephalic DA regions are reciprocally connected with the MR. MR afferents originating in the ventral tegmental area, substantia nigra, retrorubral fields, and rostral and caudal linear nuclei were observed in the present study. The dopaminergic nature of these afferents remain to be elucidated.

The dependence of the MR-neurokinin induced hyperactivity on ascending mesotelencephalic DA systems has yet to be evaluated. However, the behavioral evidence obtained in the present study suggests that the hyperactivity elicited is mediated by 5-HT-DA interactions in one of these systems. First, the hyperactivity produced by activation of an NK-3 receptor was not affected by destruction of ascending 5-HT fibers rostral to the mesencephalon, but was blocked by destruction of 5-HT neurons within the MR. Therefore, a 5-HT projection to either the ventral tegmental area or substantia nigra may mediate intra-raphé neurokinin induced LMA. Secondly, pretreatment with the DA antagonist, haloperidol, can block the effects of DiMe-C7. The 5-HT innervation of

the substantia nigra has been proposed to originate in the MR (Dray et al., 1976, van der Kooy and Hattari, 1980; Mori et al., 1987). However, recent anatomical evidence suggests that the DR is the primary source of nigral 5-HT afferents (Wirtshafter et al., 1987 b). The available anatomical evidence indicates that 5-HT neurons innervate the VTA (Herve et al., 1987; Oades and Halliday, 1987). Therefore, MR neurokinin-induced hyperactivity may be mediated by a 5-HT projection to the ventral tegmental area. In order to test this hypothesis, one could use 6-OHDA to selectively destroy DA cell bodies of the ventral tegmental area, and substantia nigra, then evaluate the effects of intra-MR neurokinins. Such studies are in progress.

#### Medial Frontal Cortical Projections and LMA

The medial aspects of the prefrontal cortex have been implicated in a number of psychological processes, including spatial problem solving, drive inhibition, and the temporal sequencing of appropriate behaviors (Brutkowski, 1965; Kolb 1974, 1984; Fuster, 1985; Herrmann et al., 1985; Vives et al., 1986). This region, furthermore, has been associated with visceral motor function (Terrebery and Neafsey, 1983). Although lesion data suggest that the medial frontal cortex modulates LMA (Kolb, 1974), it would appear that this region probably is not involved in locomotion per se. Rather, it appears to be critical for integrating spatial, temporal and environmental stimuli (see Kolb, 1984; Brutkowski, 1965; Herrmann et al., 1985; Fuster, 1985). Since the medial frontal cortex provides a rather substantial projection to the MR (this study and Leonard, 1965), it is possible that this cort-

ical region plays an important role in the processing of external stimuli during physiological and drug-induced locomotion.

The involvement of the medial frontal cortex in the regulation of an organism's response to significant environmental stimuli is further suggested by the widely reported, but little understood, stress-induced increases in frontal cortical DA metabolism (Thierry et al., 1976; Lavielle et al., 1978; Blanc et al., 1980; Bannon and Roth, 1980; Reinhard et al., 1982; Herman et al., 1982; Claustre et al., 1986; D'Angio et al., 1987). The neurokinins have been shown to regulate frontal cortical DA turnover via their modulation of DA neurons in the rostromedioventral mesencephalon (Bannon et al., 1983, 1986; Elliott et al., 1986). These changes in DA turnover are not due to locomotion per se, since rats trained to run in a wheel exhibit increases in DA metabolism in the neostriatum but not in the frontal cortex (Speciale et al., 1986). As the LMA effects of intra-raphé neurokinin administration may be mediated by DA neurons within the ventral tegmental area, it is possible that intra-MR neurokinin administration will affect DA metabolism in the frontal cortex as well.

### Conclusions

Anatomically, the MR is in a position both to modulate and to be modulated by a number of regions involved in the regulation of motor function. The heterogeneity of the locomotor responses observed in the present study reflects the complexity and diversity of the afferent modulation of MR neurons and their projections.

## SUMMARY

The overall objective of the dissertation project was to investigate the origin and functional role of the neurokinin innervation of the median raphe nucleus (MR) in the rat. The neurokinins (SP, NKA, and NKB) are found in mammals and are members of a family of structurally related bioactive peptides called tachykinins. The non-mammalian tachykinins include eledoisin, kassinin, and physalaemin. The neurokinins have a distinct distribution of binding sites (NK-1, NK-2, NK-3) in the CNS. The neurokinins and their recognition sites, furthermore, have been localized within the midbrain raphe nuclei. The specific aims of the project were to determine: 1) the behavioral effects of direct injection of the neurokinins into the MR; 2) which neurokinin(s) is (are) involved; 3) if the behavioral effects depend on the integrity of raphe 5-HT neurons; and, 4) the origin of the neurokinin innervation of the MR.

Our laboratory was the first to show that intra-raphé injections of a neurokinin agonist, the metabolically stable SP analogue, DiMe-C7, produce dose-dependent increases in locomotor activity which are dependent upon intact midbrain 5-HT neurons. This neurokinin induced behavioral activation can be blocked by administration of the dopamine (DA) antagonist, haloperidol. In the present study, the dose-dependent behavioral effects of the mammalian and non-mammalian tachykinins and the neurokinin B agonists, DiMe-C7 and senktide, were compared. Unfortunately, non-neurotoxic antagonists currently are not available so

that the neurokinin recognition site(s) involved could be further characterized.

The dose-response analyses suggested that behavioral arousal can be induced by activation of MR NK-3 sites with senktide and DiMe-C7, and by activation of MR NK-2 sites with NKA. Intra-raphe injections of the serotonergic neurotoxin, 5,7-DHT, prevented the hyperactivity produced by infusions of DiMe-C7, NKA and senktide into the MR. In contrast, previous studies demonstrated that similar lesions did not block the hyperactivity induced by intra-raphe administration of the GABA<sub>A</sub> agonist, muscimol. 5,7-DHT lesions of the 5-HT fibers which ascend in the median forebrain bundle, rostral to the mesencephalic DA cell groups, however, did not block the increases in activity produced by senktide or DiMe-C7, but did attenuate the hyperactivity produced by NKA. These results suggest that the increases in locomotor activity induced by senktide may be mediated by a 5-HT projection to the ventral tegmental area and/or the substantia nigra, while the effects produced by NKA may be mediated, in part, by 5-HT fibers which innervate the forebrain. Additional studies are needed in order to confirm this hypothesis.

Analysis of the animals' behavior in an open field arena led to a further differentiation of the effects of senktide and NKA following their injection into the MR. Senktide produced elevated activity levels when the animals were placed in a novel environment, while NKA induced an increase in exploration only in a familiar environment. Thus, it is possible that neurokinin--5-HT interactions in the MR may regulate emotional tone and motivational processes. The behavioral

arousal induced by intra-raphé muscimol administration is associated with feeding behavior and increases in plasma ACTH and corticosterone levels which are not dependent on intact 5-HT neurons. In contrast, the behavioral activation produced by infusions of either DiMe-C7 or senktide into the MR are not accompanied by either feeding or changes in plasma hormone concentrations. It remains to be seen if intra-raphé injections of NKA, or a specific NK-1 agonist, such as septide, would affect ingestive behaviors or pituitary-adrenal function.

Retrograde tracing studies (utilizing the fluorescent dye, Fluoro-Gold) were performed in order to determine the origins of the afferent projections to the MR. Several of the brain regions found to project to the MR have been implicated in the regulation of motor function. Although further studies are necessary, these data provide an anatomical basis for the possible role of the MR in the initiation, sequencing, and maintenance of distinct behavioral patterns.

Fluorescence immunocytochemistry and HPLC-RIA confirmed previous reports that fibers containing neurokinin-like substances are located within the midbrain raphe. However, combined neurokinin immunocytochemistry with Fluoro-Gold retrograde tracing did not reveal the origin(s) of the neurokinin input to the MR. Brain stem regions were found which contained both retrogradely labeled cells and neurokinin immunoreactive neurons, namely, the dorsal raphe nucleus, the interpeduncular nucleus, the rostral central gray, and the laterodorsal tegmental nucleus. Unfortunately, clear-cut evidence for double labeling in these structures was not obtained. The neurokinin neurons which project to the MR remain to be demonstrated.

The immunocytochemical studies indicated, furthermore, the need to refine the approach used to visualize neurokinin cell bodies in various CNS regions. Further studies should include the use of intracerebral injections of colchicine to arrest axonal transport, and the development of histochemical techniques which would optimize the staining of neurokinin-like substances in distinct brain regions.

Future studies also should examine the electrophysiological effects of the neurokinins on 5-HT and non-5-HT neuronal activity in the midbrain raphe. This would allow a more complete understanding of the physiology of raphe neurokinin/5-HT interactions.

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**APPENDIX I**

## Peptide Analysis and Preparation

Documentation concerning the purity and content of each peptide was supplied. Most of the batches contained > 84% of the peptide. In these cases, no corrections were made for determination of molar concentrations. One batch of neurokinin A, however, had a peptide content of only 76%. A correction was made to obtain the proper stock solution, as noted below.

1.) Vehicle: 0.1 M phosphate buffered saline (PBS) with 0.01 M acetic acid added, pH 7.2 (350 ml PBS and 150 ml 0.01 M acetic acid)

2.) Preparation of Stock Solutions:

DiMe-C7 (MW 880.13):

5.0 mg lyophilized peptide is dissolved in 250  $\mu$ l 0.01 M acetic acid. Once dissolved, 585  $\mu$ l PBS is added.

Stock concentration = 6.0  $\mu$ g/ $\mu$ l = 7.0 nmol/ $\mu$ l

Senktide (MW 842.1):

1.0 mg lyophilized peptide dissolved in 400  $\mu$ l 0.01 M acetic acid, followed by 100  $\mu$ l PBS.

OR: 5.0 mg dissolved in 2000  $\mu$ l acetic acid followed by 500  $\mu$ l PBS.

Stock concentration = 2.0  $\mu$ g/ $\mu$ l = 2.4 nmol/ $\mu$ l

Neurokinin A (MW 1133.48):

Batch 1: 5.0 mg lyophilized peptide dissolved in 300  $\mu$ l 0.01 M acetic acid, followed by 535  $\mu$ l PBS.

Batch 2: Since the biochemical analysis provided by the manufacturer indicated < 80% purity (see below), a correction was made:

5.0 mg supplied (0.76) = 3.8 mg actual, dissolved in 200  $\mu$ l 0.01 M acetic acid followed by 435  $\mu$ l PBS.

Stock concentration = 6.0  $\mu$ g/ $\mu$ l = 5.3 nmol/ $\mu$ l

Substance P (MW 1347.80):

5.0 mg lyophilized peptide was dissolved in 1000  $\mu$ l 0.01 M acetic acid, followed by 500  $\mu$ l PBS.

Stock concentration = 3.3  $\mu$ g/ $\mu$ l - 2.4 nmol/ $\mu$ l

Eledoisin (MW 1188.57):

5.0 mg lyophilized peptide dissolved in 1300  $\mu$ l 0.01 M acetic acid, followed by 235  $\mu$ l PBS.

Stock concentration = 3.3  $\mu$ g/ $\mu$ l - 2.8 nmol/ $\mu$ l

Kassinin (MW 1334.74):

5.0 mg lyophilized peptide dissolved in 1200  $\mu$ l 0.01 M acetic acid, followed by 235  $\mu$ l PBS.

Stock concentration = 3.5  $\mu$ g/ $\mu$ l - 3.1 nmol/ $\mu$ l

Physalaemin (MW 1265.61):

5.0 mg lyophilized peptide was dissolved in 250  $\mu$ l 0.01 M acetic acid, followed by 535  $\mu$ l PBS.

Stock concentration = 6.0  $\mu$ g/ $\mu$ l - 4.7 nmol/ $\mu$ l

Neurokinin B (MW 1210.58):

4.0 mg lyophilized peptide dissolved in 100  $\mu$ l, 88% formic acid, followed by 800  $\mu$ l 9.8 mM PBS, pH 11.0 (final pH 3.8).

Stock concentration = 4.4  $\mu$ g/ $\mu$ l - 3.6 nmol/ $\mu$ l

Vehicle: 10 ml 88% formic acid and 80 ml PBS (pH 11.0).  
Final pH = 3.8.

Note: Peptide stock solutions were divided into aliquots and stored in polyethylene tubes frozen at 0°C. On a drug day, the peptides were thawed, and appropriate molar dilutions made. All solutions were kept cool and dark until used.

3.) Manufacturers Purification and Content Analysis:

DiMe-C7 (Peninsula Laboratories, Belmont CA): All DiMe-C7 used was from the same lot number, although purchased separately.

Code 7473



Lot 011045

5mg

[pGlu<sup>5</sup>, MePhe<sup>8</sup>, Sar<sup>9</sup>]  
(Substance P 5-11)

811 Taylor Way, Belmont, Ca 94002 (415) 582-5362

**PENINSULA LABORATORIES, INC.**

KEEP FROM LIGHT AND DRY

For Research Purposes Only  
Not For Use In Humans**QUALITY CONTROL RECORD**  
**PENINSULA LABORATORIES, INC.**

811 TAYLOR WAY, BELMONT, CALIF. 94002 • (415) 582-5362 • (800) 827-1616 • TELEX NO. 173611

Catalog No. 7473

Date: September 29, 1986

Name: [pGlu<sup>5</sup>, Me-Phe<sup>8</sup>, Sar<sup>9</sup>] Substance P (5-11)

Lot No.: 011045

Sequence: pGlu-Gln-Phe-(Me-Phe)-Sar-Leu-Met-NH<sub>2</sub>

M. W.: 880.13

**LOT ANALYSIS:****THIN LAYER CHROMATOGRAPHY**Silica F (nBuOH:Pyr:HOAc:H<sub>2</sub>O, 15:10:3:12) o-tolidineResults: Single spot running with the standard. R<sub>f</sub> = 0.67Silica Fm (nBuOH:Pyr:HOAc:H<sub>2</sub>O, 15:15:3:12) o-tolidineResults: Single spot running with the standard. R<sub>f</sub> = 0.67**ELECTROPHORESIS**

Whatman 3 MM; pH 1.9(HCOOH:Acet); 1000 V, 1 hr.; o-tolidine

Results: Single spot traveling with the standard toward the cathode.  
R<sub>f</sub> = 0.07 with reference to Arginine.**AMINO ACID ANALYSIS**Chromatogram # 63-1402 92.6% peptide

	Theory	Found
NH <sub>3</sub>	2	1.81
Glu	2	1.94
Met	1	1.00
Leu	1	0.99
Phe	1	1.06
Sar	1	0.71
NMe-Phe	1	- *

\* The value of NMe-Phe is not determined by Amino Acid Analysis.

Note: All peptides should be stored in a cool &amp; dry place until in use.

Certified by: G. Citrowsky

Quality Control Manager

D.M.C-7

HPLC MODEL Beckman - 344 CHROMAT. NO. B-11077

COMPOUND [ $C_{12}H_{15}N_2O_2$ ,  $M_r 241.3$ ,  $S_{at} 9$ ] CODE 7473

Substance P (4-11)

Fresh  $10 \times 9016$ 

COLUMN C-18 PRESSURE psig. FLOW 1 ml/min

SOLVENT: A 0.1% TFA B 60%  $CH_2CN$  in A

GRADIENT: linear (curve) ; 0 to 100% B in 40 min

RECORDER 2 mm /min. DETECTOR 210 nm 2 AUFS

SAMPLE 0.22 mg in 40  $\mu$ l of A (plus 0  $\mu$ l of B)INJECT 20  $\mu$ l OPERATOR SM DATE 10-1-86

HPLC MODEL Altex - 332 CHROMAT. NO. S-11087

COMPOUND [ $C_{12}H_{15}N_2O_2$ ,  $M_r 241.3$ ,  $S_{at} 9$ ] CODE 7473

Substance P (5-11)

 $10 \times 9016$ 

COLUMN C-18 PRESSURE psig. FLOW 1 ml/min

SOLVENT: A 0.05M  $NaH_2PO_4$  B 60%  $CH_2CN$  in A

GRADIENT: linear (curve) ; 0 to 100% B in 40 min

RECORDER 2 mm /min. DETECTOR 210 nm 1 AUFS

SAMPLE 0.22 mg in 40  $\mu$ l of A (plus 0  $\mu$ l of B)INJECT 20  $\mu$ l OPERATOR SM, SF DATE 10-1-86

Senktide (Bachem, Torrance, CA): All Senktide used was from the same lot number, although purchased separately.

b

**BACHEM** INC.  
FINE CHEMICALS

### Certificate of Analysis

PRODUCT: Senktide

LOT NUMBER: 655C

RELEASE DATE: December 1986

STRUCTURE: Succinyl-Asp-Phe-N-MePhe-Gly-Leu-Met-NH<sub>2</sub>

MOLECULAR WEIGHT: 842.1

APPEARANCE: White powder

AMINO ACID ANALYSIS:

Asx 0.95	Thr ---	Ser ---
Glx ---	Pro ---	Gly 1.00
Ala ---	Cys ---	Val ---
Met 1.00	Ile ---	Leu 1.00
Tyr ---	Phe 0.97*	His ---
Lys ---	Arg ---	Trp ---

\*N-MePhe = Present, but not quantitated

PEPTIDE CONTENT: 83.8% (+3%)

PEPTIDE PURITY: >99% (See attached HPLC trace)

  
BACHEM, INC.

BACHEM INC.  
5125 KLASOWA STREET, TORRANCE, CA 90505  
310/530-4171 TWX 510-517-7230 BACHEM TRNC

BACHEM UK  
50 HIGH STREET, SAFFRON WALDEN, Essex CB10 1AA  
TELE (0799) 26-06 TEL 01726 BACHEM 0

SENK TIDE

START

MAC

SAMPLE SENK TIDE Lot# 635C  
281 mg / 100 ml + 10 ml / 100 ml 2g / 100 ml  
 SOLVENTS: MTBE CH2Cl2  
 GRADIENT B 20 25% / 15  
 COLUMN: Vyadsorb 1720.49 # 20  
 SET D: 0.16 2791 ATIC 4  
 MIN. AREA: 500 FLOW: 1.6 ML/M  
 DONE BY: JW DATE: 11-28-80

22.977

22.032

CHROMATOPAC C-RSA  
 SAMPLE NO 8  
 REPORT NO 394

FILE 1  
 METHOD 41

PKNO	TIME	AREA	PK	IDNO	CONC	NAME
1	22.032	671086			99.8021	
2	22.977	1336			0.1979	
TOTAL		672418			100	

221-22412  
 6030100  
 078

Neurokinin A (Peninsula and Bachem):

## QUALITY CONTROL RECORD

### PENINSULA LABORATORIES, INC.

811 TAYLOR WAY, BELMONT, CALIF. 94002 • (415) 597-6392 • (800) 927-1516 • TELEX NO. 172511

Catalog No. 7359

Date: June 17, 1986

Name:  $\alpha$ -Neurokinin (Neuromedin L, Substance K)

Lot No.: 010251

Sequence: His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH<sub>2</sub>

M. W.: 1133.48

**LOT ANALYSIS:**Thin Layer Chromatography:Cellulose F: nBuOH:Pyr:HOAc:H<sub>2</sub>O 15:10:3:12      NinhydrinSilica nBuOH:EtOAc:HOAc:H<sub>2</sub>O 1:1:1:1      NinhydrinSilica Fm: nBuOH:Pyr:HOAc:H<sub>2</sub>O 6:6:1.2:4.8      NinhydrinResults: Fm: Single spot. R<sub>f</sub> = 0.56F: Single spot. R<sub>f</sub> = 0.75Electrophoresis:

Whatman 3MM Pyr:Acet., pH3.5; 1500V., 1 hr.      Ninhydrin

Results: Single spot traveling towards the cathode.

R<sub>f</sub> = 0.60 with reference to arginine.AMINO ACID ANALYSIS:

Chromatogram No. 63-0214

% of Peptide 88.7%

	Theory	Found		Theory	Found
Lys	1	1.00	Gly	1	0.98
His	1	0.81	Val	1	0.91
NH <sub>2</sub>	1	1.09	Met	1	1.07
Asp	1	1.02	Leu	1	1.08
Thr	1	0.95	Phe	1	0.95
Ser	1	1.04			

Certified by: G. Citovsky

Quality Control Manager

Code 7359



Lot 010251

5mg

Neurokinin A (Neuromedin L,  
Substance K)

811 Taylor Way, Belmont, Ca. 94002 (415) 597-6392

**PENINSULA LABORATORIES, INC.**

KEEP COOL AND DRY

For Research Purposes Only  
Not For Use in Humans

HPLC MODEL Beckman - 344 CHROMAT. NO. B-8664COMPOUND Neurokinin A CODE 7359 F-4-9

R.F.

LOT 108429COLUMN C-18 PRESSURE \_\_\_\_\_ psig. FLOW 1 ml/minSOLVENT: A 0.1% TFA B 60% CH<sub>3</sub>CN in AGRADIENT: linear (curve \_\_\_\_\_) ; 0 to 100% B in 40 minRECORDER 2 mm /min. DETECTOR 210 mm 2 AUFSSAMPLE 0.2 mg in 400  $\mu$ l of A (plus 0  $\mu$ l of B)INJECT 8  $\mu$ l OPERATOR SM, JD DATE 6-11-86HPLC MODEL Altex - 332 CHROMAT. NO. B-8677COMPOUND Neurokinin A CODE 7359 F-4-9

R.F.

LOT 108429COLUMN C-18 PRESSURE \_\_\_\_\_ psig. FLOW 1 ml/minSOLVENT: A 0.05M NaH<sub>2</sub>PO<sub>4</sub> B 60% CH<sub>3</sub>CN in AGRADIENT: linear (curve \_\_\_\_\_) ; 0 to 100% B in 40 minRECORDER 2 mm /min. DETECTOR 210 mm 1 AUFSSAMPLE 0.2 mg in 400  $\mu$ l of A (plus 0  $\mu$ l of B)INJECT 60  $\mu$ l OPERATOR SM, JD DATE 6-11-86

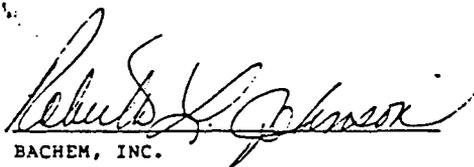
**b****BACHEM** INC.  
FINE CHEMICALS

## Certificate of Analysis

PRODUCT: Substance K (alpha-Neurokinin)LOT NUMBER: 546BRELEASE DATE: December 1985STRUCTURE: H<sub>2</sub>N-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH<sub>2</sub>MOLECULAR WEIGHT: 1133.5APPEARANCE: White powderAMINO ACID ANALYSIS:

Asx	1.00	Thr	1.00	Ser	0.68*
Glx	---	Pro	---	Gly	1.01
Ala	---	Cys	---	Val	1.04
Met	0.98	Ile	---	Leu	0.99
Tyr	---	Phe	1.00	His	0.99
Lys	1.02	Arg	---	Trp	---

\*Partially destroyed during acid hydrolysis

PEPTIDE CONTENT: 76.48% (+3%)PEPTIDE PURITY: >99% (See attached HPLC trace)


BACHEM, INC.

BACHEM INC.  
3132 KASHIWA STREET, TORRANCE, CA 90505  
213/606-4171 TWX 910-347-7299 BACHEM TRAC

BACHEM UK  
20 HIGH STREET, SAFFRON WALDEN, Essex CB10 1AA  
TELE (0779) 26446 Tlx 917294 BACHEM G

**b** **BACHEM** INC.  
FINE CHEMICALS  
3132 Kashiwa Street  
Torrance, Calif. 90505

α-Neurokinin (Substance K,  
Neuromedin L)

QTY 5MG

LOT # 546B

NOT FOR HUMAN USE

alpha-Neurokinin, Substance K, Neromedin L

lot#546B

12/3/85

Dr. Goud

12/3/85

< Neromedin  
 70 B 15 <sup>25</sup> 40  
 Flow 1.5ml/min  
 210nm  
 A-TEAP P-H 2-3  
 B-CH<sub>3</sub>CN

START 00.00.00.00.

ATTEN

11.63

STOP

C-R1B  
 SMPL # 00  
 FILE # 1  
 REPT # 5065  
 METHOD 41

#	NAME	TIME	CONC	MK	AREA
0		10.96	99.4442		122219
0		11.63	0.5557	V	682
	TOTAL		100		122902

Substance P (Peninsula):

QUALITY CONTROL RECORD  
PENINSULA LABORATORIES, INC.

611 TAYLOR WAY, BELMONT, CALIF. 94002 • (415) 582-5382 • (800) 822-1818 • TELEX NO. 172511

Catalog No. 7451 Date: February 17, 1987  
Name: Substance P Lot No.: 011989  
Sequence: Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH<sub>2</sub> M. W.: 1347.80

## LOT ANALYSIS:

THIN LAYER CHROMATOGRAPHY:

Silica F (nBuOH:Pyr:HOAc:H<sub>2</sub>O, 15:10:3:12) Ninhydrin  
Silica Fc (nBuOH:Pyr:HOAc:H<sub>2</sub>O, 21:12:2:15) Ninhydrin  
Silica Fm (nBuOH:Pyr:HOAc:H<sub>2</sub>O, 15:15:3:12) Ninhydrin  
Results: F Single spot running slightly behind the standard. Rf = 0.36  
Fc Single spot running slightly behind the standard. Rf = 0.29  
Fm Single spot running with the standard. Rf = 0.54

ELECTROPHORESIS:

Whatman 3MM, pH 3.5(Pyr:Acet.); 1500V. 1hr. Ninhydrin  
Results: Single spot running with the standard toward the cathode.  
Rf=0.76 with reference to Arginine. 1

AMINO ACID ANALYSIS:

Chromatogram No. 63-2775 % of peptide 86.3Z

	Theory	Found
Lys	1	1.04
NH <sub>3</sub>	3	2.91
Arg	1	1.18
Glu	2	2.08
Pro	2	1.97
Gly	1	0.91
Met	1	0.85
Leu	1	1.01
Phe	2	1.94

Certified by: *B. Citovsky*

Quality Control Manager

Note: All peptides should be stored in  
a cool & dry place until in use.

HPLC MODEL Beckman 344 CHROMAT. NO. 8-17613  
 COMPOUND Substance P CODE 7451  
748-83 10 X09824  
 COLUMN PRESSURE psig. FLOW ml/min  
 SOLVENT A 0.17FA 60/CH3CN  
 GRADIENT (curve) to 70 in min  
 RECORDER In/min DETECTOR cm IAUFS  
 SAMPLE 0.23 mg in 400 ul of ALIQUOT LIT. SUBJECT S-11  
 OPERATOR SF DATE 2-10-87

HPLC MODEL Altex 332 CHROMAT. NO. 8-17613  
 COMPOUND Substance P CODE 7451  
748-83 10 X09824  
 COLUMN G-18 PRESSURE psig. FLOW ml/min  
 SOLVENT A 0.17FA 60/CH3CN  
 GRADIENT (curve) to 70 in min  
 RECORDER In/min DETECTOR cm IAUFS  
 SAMPLE 0.23 mg in 400 ul of ALIQUOT LIT. SUBJECT S-11  
 OPERATOR SF DATE 2-10-87

Code 7451

Lot 011989



5mg

Substance P

KEEP COOL AND DRY

For Research Purposes Only Not for Use in Humans

811 Taylor Way, Belmont, CA 94002 (415) 882-6282

**PENINSULA LABORATORIES, INC.**

Neurokinin B (Peninsula):

QUALITY CONTROL RECORD  
PENINSULA LABORATORIES, INC.

811 TAYLOR WAY, BELMONT, CALIF. 94002 • (415) 592-5392 • (800) 922-1816 • TELEX NO. 172511

Catalog No. 7357 Date: March 28, 1985  
Name: Neurokinin B (Neuromedin K, Neurokinin B) Lot No.: 007472  
Sequence: Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH<sub>2</sub> M.W.: 1210.58  
Note: Dissolve in 88% formic acid and dilute with desired buffer.

## LOT ANALYSIS:

Thin Layer Chromatography:

Silica nBuOH:EtOAc:HOAc:H<sub>2</sub>O (1:1:1:1) Ninhydrin

Results: Single spot migrating the same as the standard.

Electrophoresis:

Whatman 3MM HCOOH:Acet., pH 1.9; 1000V., 1 hr. Ninhydrin

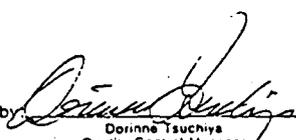
Results: Main spot with tailing.

$R_f = 0.42$  with reference to arginine.

AMINO ACID ANALYSIS:

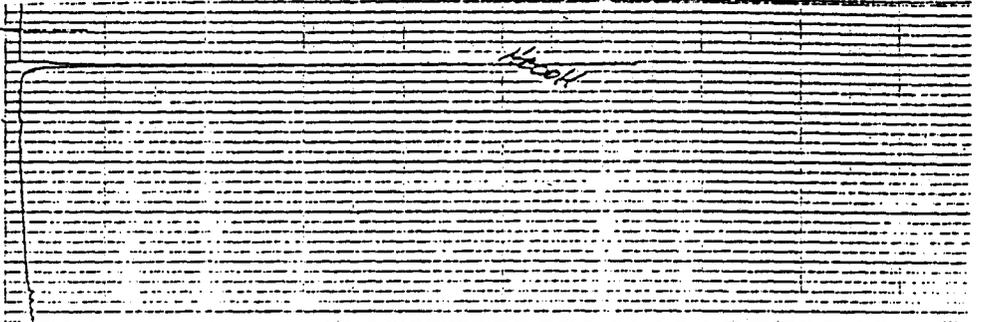
Chromatogram No. CL-7108 % of Peptide 94%

	Theory	Found
His	1	1.04
NH <sub>2</sub>	1	1.28
Asp	2	1.98
Gly	1	1.02
Val	1	1.03
Met	2	2.08
Leu	1	0.93
Phe	2	2.06

Certified by 

Dorinne Tsuchiya  
Quality Control Manager

HPLC MODEL Boekman 344 CHROMAT. NO. B-0987  
 COMPOUND  $\beta$ -Neurokinin CODE 7357  
75-77 LOT X06523  
 COLUMN C-18 PRESSURE \_\_\_\_\_ psig. FLOW 1 ml/min  
 SOLVENT : A 0.1% TFA B 60% CH<sub>2</sub>OH in A  
 GRADIENT Linear (curve ); 0 to 100% B in 40 min  
 RECORDER 2 mV/min DETECTOR 210nm 2 AUFS  
 SAMPLE 46  $\mu$ g in 400  $\mu$ l of A (plus  $\mu$ lB); Inject 15  $\mu$ l  
 OPERATOR F. J. Skoch DATE 3/28/85



HPLC MODEL Altex 332 CHROMAT. NO. B-0977  
 COMPOUND  $\beta$ -Neurokinin CODE 7357  
75-77 Re. Std. 007472 LOT X06523  
 COLUMN C-18 PRESSURE \_\_\_\_\_ psig. FLOW 1 ml/min  
 SOLVENT : A 0.05M NaH<sub>2</sub>PO<sub>4</sub> B 60% CH<sub>2</sub>OH in A  
 GRADIENT Linear (curve ); 0 to 100% B in 40 min  
 RECORDER 2 mV/min DETECTOR 210nm 1 AUFS  
 SAMPLE 24  $\mu$ g in 300  $\mu$ l of A (plus  $\mu$ lB); Inject 10  $\mu$ l  
 OPERATOR F. J. Skoch DATE 3/28/85

Code 7357 Lot 007472  
  
 5mg  
 Neurokinin B  
 (Neuromedin K)  
 811 Taylor Way, Belmont, Ca 94007 (415) 862-4392  
**PENINSULA LABORATORIES, INC.**

KEEP COOL AND DRY

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Eledoisin (Peninsula):

## QUALITY CONTROL RECORD

### PENINSULA LABORATORIES, INC.

811 TAYLOR WAY, BELMONT, CALIF. 94002 • (415) 597-5392 • (800) 922-1516 • TELEX NO. 172511

Catalog No. 7101		Date: October 9, 1986
Name: Eledoisin		Lot No.: 011073
Sequence: pGlu-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH <sub>2</sub>		M.W.: 1188.57

**LOT ANALYSIS:**Thin Layer Chromatography:Cellulose Fc: nBuOH:Pyr:HOAc:H<sub>2</sub>O 42:24:4:30      o-tolidineSilica Fm: nBuOH:Pyr:HOAc:H<sub>2</sub>O 6:6:1.2:4.8      o-tolidineResults: Fc: Single spot running slightly ahead of the standard.  $R_f = 0.71$ Fm: Single spot running with the standard.  $R_f = 0.64$ Electrophoresis:

Whatman 3MM HCOOH:Acet., pH 1.9; 1000V., 1 hr.      o-tolidine

Results: single spot running with the standard towards the cathode.

 $R_f = 0.24$  with reference to Arginine.AMINO ACID ANALYSIS:Chromatogram No. 63-1439      % of Peptide 90.5%

	Theory	Found		Theory	Found
Lys	1	0.98	Gly	1	1.05
NH <sub>3</sub>	1	1.60	Ala	1	1.03
Asp	1	0.95	Met	1	0.99
Ser	1	0.93	Ile	1	1.00
Glu	1	1.04	Leu	1	1.04
Pro	1	0.96	Phe	1	0.95

Certified by: E. Cibulsky  
Quality Control Manager

NOTE: Peptides should be stored in a cool & dry area until in use.

HPLC MODEL Beckman - 344 CHROMAT. NO. B-11121COMPOUND Eledoisin CODE 7101FreshA.M.  
Lot X09038COLUMN C-18 PRESSURE \_\_\_\_\_ psig. FLOW 1 ml/minSOLVENT: A 0.12 TFA B 60% CH<sub>3</sub>CN in AGRADIENT: linear (curve \_\_\_\_\_) ; 0 to 100% in 40 minRECORDER 2 mm /min. DETECTOR 210 nm 2 AUFSSAMPLE 0.26 mg in 400  $\mu$ l of A (plus 0  $\mu$ l of B)INJECT 12  $\mu$ l OPERATOR SM, SE DATE 10-2-86HPLC MODEL Altex - 332 CHROMAT. NO. B-11109COMPOUND Eledoisin CODE 7101FreshA.M.  
Lot X09038COLUMN C-18 PRESSURE \_\_\_\_\_ psig. FLOW 1 ml/minSOLVENT: A 0.05M NaH<sub>2</sub>PO<sub>4</sub> B 60% CH<sub>3</sub>CN in AGRADIENT: linear (curve \_\_\_\_\_) ; 0 to 100% in 40 minRECORDER 2 mm /min. DETECTOR 210 nm \_\_\_\_\_ AUFSSAMPLE 0.26 mg in 400  $\mu$ l of A (plus 0  $\mu$ l of B)INJECT 60  $\mu$ l OPERATOR SM, SE DATE 10-7-86

Code 7101

Lot 011073

**2**

5mg

Eledoisin

011 Turner Way, Belmont, Ca 94002 (415) 867-4392

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For Research, Diagnostic, and  
Pharmaceutical Use in Humans

Kassinin (Peninsula):

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PENINSULA LABORATORIES, INC.

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Catalog No. 7111 Date: September 19, 1986  
Name: Kassinin Lot No.: 010902  
Sequence: Asp-Val-Pro-Lys-Ser-Asp-Gln-Phe-Val-Gly-Leu-Met-NH<sub>2</sub> M. W.: 1334.74

LOT ANALYSIS:Thin Layer Chromatography:

Silica Fm: nBuOH:Pyr:HOAc:H<sub>2</sub>O 6:6:1.2:4.8 Ninhydrin

Silica nBuOH:EtOAc:HOAc:H<sub>2</sub>O 1:1:1:1 Ninhydrin

Results: Fm: Main spot running with the standard with a faint lower and negligible upper spot. R<sub>f</sub> = 0.54

1:1:1:1 Main spot running with the standard with a faint lower spot. R<sub>f</sub> = 0.50

Electrophoresis:

Whatman 3MM HCOOH:Acet., pH 1.9; 1000V., 1 hr. Ninhydrin

Results: Single spot running with the standard towards the cathode.

R<sub>f</sub> = 0.36 with reference to Arginine.

AMINO ACID ANALYSIS:

Chromatogram No. 63-1242

% of Peptide 95.17

	Theory	Found		Theory	Found
Lys	1	1.07	Pro	1	1.09
NH <sub>2</sub>	2	2.05	Gly	1	1.00
Asp	2	1.78	Val	2	2.00
Ser	1	0.89	Met	1	1.10
Glu	1	1.06	Leu	1	1.08
			Phe	1	0.99

Certified by: 6. Citovsky

Quality Control Manager

NOTE: Peptides should be stored in a cool & dry area until in use.

HPLC MODEL Beckman - 344 CHROMAT. NO. B-10647

COMPOUND Kassinin CODE 7111FreshR.M.  
Lot X08931COLUMN C-18 PRESSURE \_\_\_\_\_ psig. FLOW 1 ml/minSOLVENT: A 0.1% TFA B 60% CH<sub>3</sub>CN in AGRADIENT: linear (curve \_\_\_\_\_) ; 0 to 100% B in 40 minRECORDER 2 mm /min. DETECTOR 210 nm 2 AU'sSAMPLE 0.25 mg in 400  $\mu$ l of A (plus 0  $\mu$ l of B)INJECT 6  $\mu$ l OPERATOR G.C. DATE 9-15-86HPLC MODEL Altex - 332 CHROMAT. NO. B-10653COMPOUND Kassinin CODE 7111FreshR.M.  
Lot X08931COLUMN C-18 PRESSURE \_\_\_\_\_ psig. FLOW 1 ml/minSOLVENT: A 0.05M NaH<sub>2</sub>PO<sub>4</sub> B 60% CH<sub>3</sub>CN in AGRADIENT: linear (curve \_\_\_\_\_) ; 0 to 100% B in 40 minRECORDER 2 mm /min. DETECTOR 210 nm 1 AU'sSAMPLE 0.25 mg in 400  $\mu$ l of A (plus 0  $\mu$ l of B)INJECT 50  $\mu$ l OPERATOR G.C. DATE 9-15-86

Code 7111



Lot 010902

5mg

Kassinin

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Catalog No. 7110

Date: April 16, 1986

Name: Physalaemin

Lot No.: 009870

Sequence: pGlu-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH<sub>2</sub>

M. W.: 1265.61

LOT ANALYSIS:Thin Layer Chromatography:Silica nBuOH:EtOAc:HOAc:H<sub>2</sub>O 1:1:1:1                      NinhydrinSilica F: nBuOH:Pyridine:HOAc:H<sub>2</sub>O 15:10:3:12                      NinhydrinSilica Fm: nBuOH:Pyridine:HOAc:H<sub>2</sub>O 6:6:1.2:4.8                      NinhydrinResults: F: Single spot running with the standard. R<sub>f</sub> = 0.49          Fm: Single spot running with the standard. R<sub>f</sub> = 0.63          1:1:1:1 Single spot running with the standard. R<sub>f</sub> = 0.60Electrophoresis:

Whatman JMM Pyridine:Acetic acid, pH 6.4; 1500V., 1 hr.                      Ninhydrin

Results: Single spot running with the standard in the neutral zone.

          R<sub>f</sub> = 0.29 with reference to arginine.AMINO ACID ANALYSIS:Chromatogram No. CL-10170% of Peptide 90.3%

	Theory	Found		Theory	Found
Lys	1	1.00	Gly	1	1.02
NH <sub>3</sub>	2	3.47	Ala	1	1.05
Asp	2	1.82	Met	1	0.96
Glu	1	1.09	Leu	1	0.94
Pro	1	1.02	Tyr	1	0.95
			Phe	1	0.99

Certified by: F. Citovsky

Quality Control Manager

HPLC MODEL Beckman - 344 CHROMAT. NO. B-7546

COMPOUND Physalaemin CODE 7110

R.F. LOT X08152

COLUMN C-18 PRESSURE psig. FLOW 1 ml/min

SOLVENT: A 0.1% TFA B 60% CH<sub>3</sub>CN in A

GRADIENT: linear (curve ) ; 0 to 100% B in 40 min

RECORDER 2 mm /min. DETECTOR 210 nm 2 AUFS

SAMPLE 0.5 mg in 500 µl of A (plus 0 µl of B)

INJECT 5 µl OPERATOR SM, JD DATE 4-11-86

009870

HPLC MODEL Altex - 332 CHROMAT. NO. B-7517

COMPOUND Physalaemin CODE 7110

R.F. LOT X08152

COLUMN C-18 PRESSURE psig. FLOW 1 ml/min

SOLVENT: A 0.05% NaH<sub>2</sub>PO<sub>4</sub> B 60% CH<sub>3</sub>CN in A

GRADIENT: linear (curve ) ; 0 to 100% B in 40 min

RECORDER 2 mm /min. DETECTOR 210 nm 1 AUFS

SAMPLE 0.5 mg in 500 µl of A (plus 0 µl of B)

INJECT 30 µl OPERATOR SM, JD DATE 4-11-86

Code 7110



Lot 009870

5mg

Physalaemin

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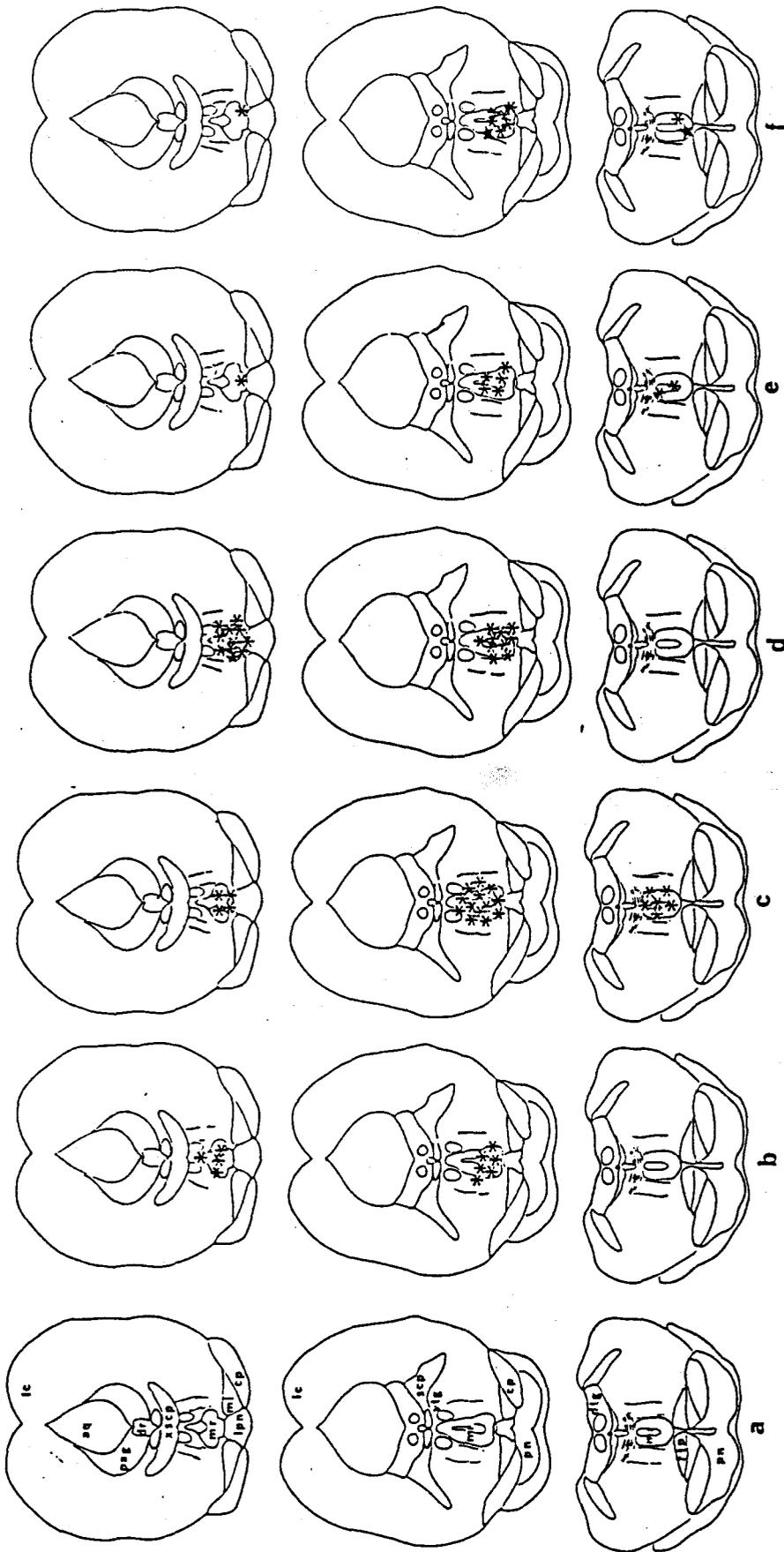
**APPENDIX II**

Cannulae PlacementsExperiments I - V: Tachykinin Dose-Responses

Fig. A.: Location of cannula tips (black stars) in the animals used in Experiments I (b) - V (f). Schematic diagrams of coronal sections separated by about 0.5 mm show the abbreviations of structures (a) and the cannula placements.

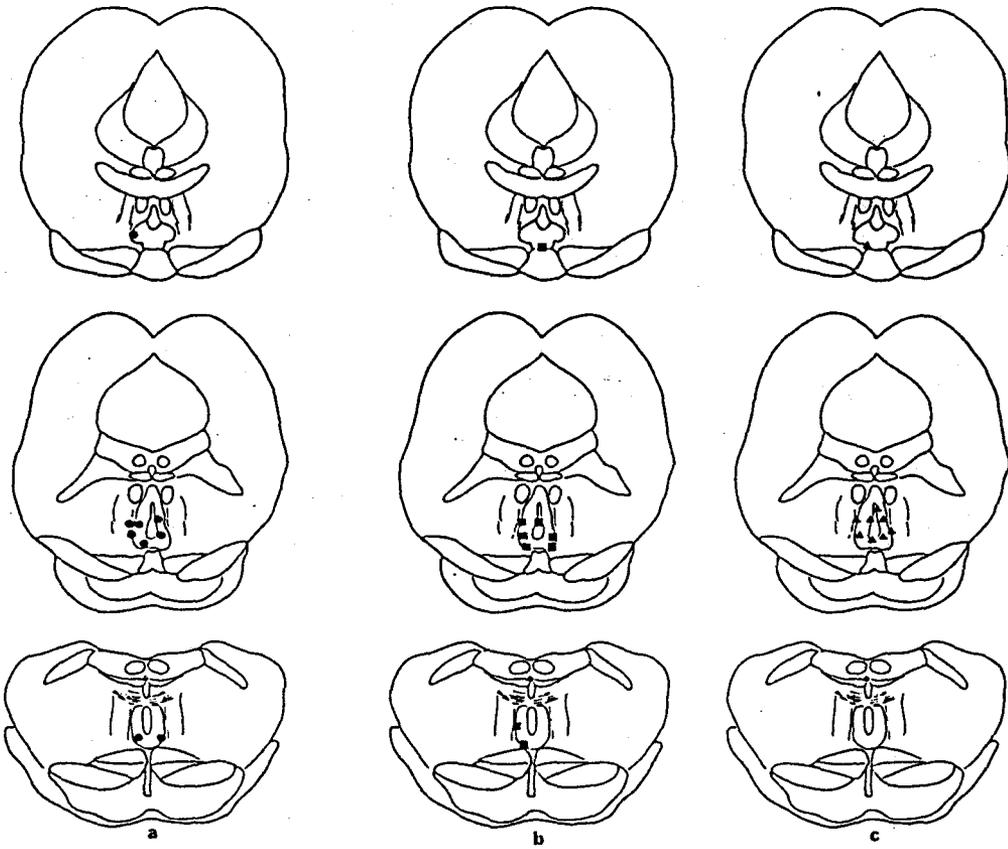
## Abbreviations:

aq: cerebral aqueduct  
cp: cerebral peduncle  
dr: dorsal raphe nucleus  
dtg: dorsal tegmental nucleus of Gudden  
ic: inferior colliculus  
ipn: interpeduncular nucleus  
ml: medial lemniscus  
mr: median raphe nucleus  
pag: periaqueductal grey  
pn: basal pontine nuclei  
rtp: reticular tegmental nucleus  
scp: superior cerebellar peduncle  
vtg: ventral tegmental nucleus of Gudden  
xscp: decussation of scp



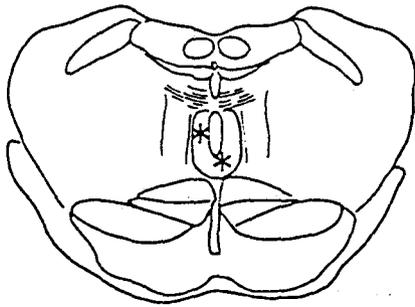
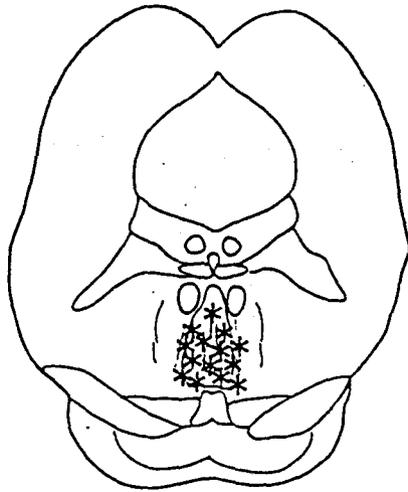
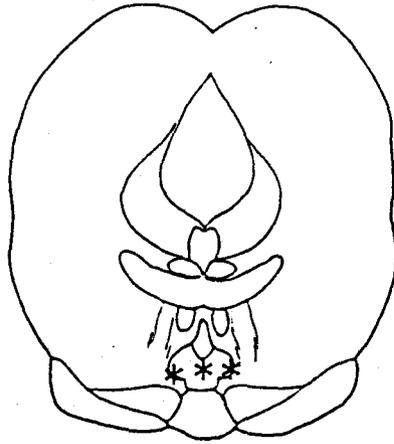
Experiment VI: MR/MFB 5.7 DHT Lesions

Fig B: Location of cannula tips in the animals used in Experiments VI. Schematic diagrams of coronal sections separated by about 0.5 mm show the abbreviations of structures in Fig. A. a) black circles - placements for control group; b) black squares - placements for MR lesion group; c) black triangles - placements for MFB lesion group.



Experiment IX: Neurokinin Habituated Open Field

Fig C: Location of cannula tips (stars) in the animals used in Experiments IX. Schematic diagrams of coronal sections separated by about 0.5 mm show the abbreviations of structures in Fig. A.



APPROVAL SHEET

The dissertation submitted by Joseph M. Paris has been read and approved by the following committee:

Dr. Stanley A. Lorens, Director  
Professor, Department of Pharmacology, Loyola University

Dr. Nae J. Dun  
Professor, Department of Pharmacology, Loyola University

Dr. T. Celeste Napier  
Assistant Professor, Department of Pharmacology, Loyola University

Dr. Edward J. Neafsey  
Associate Professor, Department of Anatomy, Loyola University

Dr. Lewis S. Seiden  
Professor, Department of Pharmacological and Physiological Sciences,  
University of Chicago

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Date

8/16 - 88

Stanley A. Lorens  
Director's Signature