STUDY OF THE NEURONAL PROJECTION FROM THE VENTRAL TEGMENTAL AREA AND SUBSTANTIA NIGRA TO THE PERIAQUEDUCTAL GRAY REGION

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STUDY OF THE NEURONAL PROJECTION FROM THE VENTRAL TEGMENTAL AREA AND SUBSTANTIA NIGRA TO THE PERIAQUEDUCTAL GRAY REGION

by

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Perfection is achieved, not when there is nothing more to add,

but when there is nothing left to take away.

Antoine de Saint-Exupery (1900-1944)

Abstract

Previous studies have shown that neurons in the ventral tegmental area (VTA) and substantia nigra (SN) project to the ventrolateral periaqueductal gray (PAGvl) and dorsal raphe nucleus (DR). Research has also shown that stimulation of neurons in the VTA/SN elicits cardiovascular depressor responses that are mediated by a projection to the PAGvl/DR. Anatomical and physiological experiments were done in the present study to determine the neurochemical identity of the VTA/SN projection to the PAGvl/DR. Experiments were done to characterize the origin and chemical nature of this projection by combining cholera toxin B tracing with immunofluorescence for the 67K isoform of glutamic acid decarboxylase (GAD) and tyrosine hydroxylase (TH). The PAGvl/DR region was found to receive a substantial input from neurons in the VTA, SN, and deep mesencephalic nucleus. The DR was preferentially innervated by neurons in the VTA whereas the PAGvl was preferentially innervated by neurons in the SN. A proportion of neurons in the VTA and the reticular part of the SN found to project to the PAGvl/DR were GAD positive. In addition, experiments were done in urethane-anesthetized rats to determine if injections of a GABA antagonist in the region of the PAGvl/DR attenuated the cardiovascular depressor responses produced by glutamate stimulation (0.01 M, 50 nl) of the VTA/SN. Injections of the GABA blocking agent picrotoxin (2.5 nmol, 500 nl) into the PAGvl/DR eliminated the cardiovascular responses from stimulation of the VTA/SN. The

results of the present investigation provide evidence for a GABAergic projection from the VTA/SN to the PAGvl/DR. This projection may be an important regulator of the PAGvl/DR, an area of the midbrain involved in the production of behavioral and physiological responses to pain and stress.

Key words: ventral tegmental area, substantia nigra, periaqueductal gray, dorsal raphe nucleus, dopamine, GABA, cardiovascular

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Chapter 1

Introduction

1.1 General Physiological Functions Associated with the Ventral Tegmental Area and Substantia Nigra

The ventral tegmental area (VTA) and substantia nigra (SN) are areas of the ventral midbrain that contain the neurotransmitter dopamine. Midbrain dopamine neurons are known to innervate many regions of the forebrain where dopamine, released from fiber terminals, functions as a neural modulator of forebrain neurotransmission (Mogenson and Yang, 1991; Yang *et al.*, 1999). The VTA and SN are also composed of other neuronal types, some of which use gamma-aminobutyric acid (GABA) and neuropeptides as neurotransmitters or modulators.

The dopaminergic projections to the forebrain have been implicated in a number of functions related to the behavioral and motor responses associated with the survival of organisms. It is generally accepted that dopamine released in the basal ganglia exerts an activating or permissive action on motor movements

(Alexander *et al.*, 1990; Afifi, 1993). The most obvious indication of dopamine's role in movement is that loss of dopamine neurons in the SN as seen in Parkinson's disease causes rigidity, hypokinesia, and tremors. Treatment with the dopamine precursor L-DOPA or with dopamine receptor agonists stops the tremors, allows movement to occur, and reduces rigidity in Parkinsonian patients. Dopamine release in the ventral striatum has also been implicated in the initiation of locomotion, and may form part of the motivational drive needed for behavior to occur (Mogenson and Huang, 1973; Mogenson and Yang, 1991). Midbrain dopamine neurons are also important for motivation and reward mediated behaviors. For example, dopamine neurons in the VTA, which innervate the ventral striatum and limbic cortex, are thought to be involved in the preparation, organization and initiation of goal-directed behaviors. In particular, dopamine neurons of the VTA are an integral part of the natural reward circuitry (Schultz, 1998, 2000, 2002), and have been implicated in the behavioral sensitization and the dependence produced by several drugs of abuse including commonly abused drugs such as heroin, alcohol, cocaine and amphetamine (Bonci *et al.*, 2003). Changes in dopamine function may be, in part, related to mood disorders such as depression and cognitive disorders such as schizophrenia (Yang *et al.*, 1999).

1.2 Possibility of Other Functions for the VTA and SN

In addition to the well-recognized influence of the dopamine neurons in the VTA and SN on movement and motivation, the VTA and SN are likely to have other important functions. It has been suggested that the VTA and SN are involved in an organism's reactivity to changes in the environment, for selective information processing and for general emotional responses, which are essential for an organism to cope with the external world (Pani *et al.*, 2000). For example, it is well known that exposure of an organism to a challenging or novel environment (so called stress) results in an increase in the metabolism and the release of dopamine in limbic structures and the basal ganglia (Finlay and Zigmond, 1997; Feenstra, 2000; Pani *et al.*, 2000). This increase in dopamine release may help in the selection of the appropriate behavioral and physiological responses to challenges in the environment (Mogenson and Yang, 1991; Kirouac and Ganguly, 1992, 1995). In addition, there are numerous studies supporting a role for both the VTA and the SN in pain modulation. Injections of morphine or other pharmacological agents into the VTA and SN have been shown to elicite antinociception in a variety of behavioral tests (Jurna *et al.*, 1978; Barnes *et al.*, 1979; Baumeister and Frye, 1986; Frye *et al.*, 1986; Baumeister *et al.*, 1987, 1988, 1989, 1990, 1993; Hebert *et al.*, 1990; Morgan and Franklin, 1990; Baumeister, 1991; Altier and Stewart, 1993, 1996, 1997, 1998), and lesions of the VTA have been shown to result in an increase of pain response or

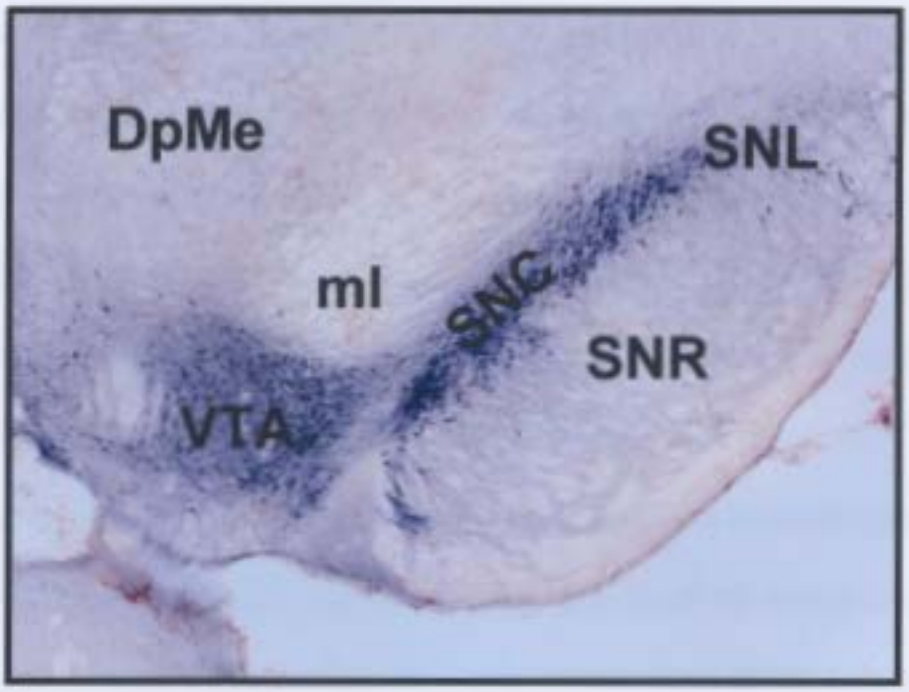
hyperalgesia (Saade *et al.*, 1997; Sotres-Bayon *et al.*, 2001). Previous studies have also shown that stimulation of the VTA and SN with the excitatory amino acid L-glutamate elicits decreases in arterial pressure (Kirouac and Ciriello, 1997b; Zhang *et al.*, 1997; Kirouac and Pittman, 2000). While speculative at this time, it is possible that the VTA and SN are involved in the regulation of physiological responses associated with motor and behavioral responses to the challenges in an organism's environment.

1.3 Anatomical Organization of the Ventral Midbrain

1.3.1 Ventral Tegmental Area and Substantia Nigra (VTA/SN)

Many of the anatomical details of the ventral midbrain are based on the location of dopamine neurons, which form the major type of neuron in this region. Figure 1 shows the location of different subdivisions of the VTA and SN through their rostrocaudal extent. The SN lies in the ventral tegmentum of the mesencephalon, and is a heterogeneous collection of neurochemically and functionally interrelated elements. Anatomically, the SN consists of three major parts (Fallon and Loughlin, 1995). The substantia nigra pars compacta (SNC) is located ventrally to the medial lemniscus and dorsally to the substantia nigra pars reticulata (SNR). The SNC is composed of a sheet of dopamine neurons that are

Fig.1. Photomicrograph of a section of the ventral midbrain showing tyrosine hydroxylase immunohistochemically positive neurons that are concentrated in the ventral tegmental area and substantia nigra. The substantia nigra is composed of the compact part where dopamine neurons are found; reticular part, which is composed mostly of GABA neurons; and lateral part, which consists of dopamine and GABA neurons. The deep mesencephalic nucleus is considered the reticular formation containing dopamine neurons mixed with GABA neurons. VTA, ventral tegmental area; SN, substantia nigra; SNC, compact part of the substantia nigra; SNR, reticular part of the substantia nigra; SNL, lateral part of the substantia nigra; DpMe, deep mesencephalic nucleus; ml, medial lemniscus.


DpMe
SNL
ml
SNC
SNR
VTA

known as the A9 dopamine cell group as first described by Dahlstrom and Fuxe (1964). The SNR is a pallidal structure formed by GABA neurons with small clusters of dopamine neurons found in various regions of the SNR. Lateral to the SNC and SNR is the substantia nigra pars lateralis (SNL), which contains another group of dopamine neurons and some non-dopaminergic neurons (Moriizumi *et al.*, 1992). As described above, the SN is populated by dopamine neurons in the SNC (Bjorklund and Lindvall, 1975) and GABA neurons in the SNR. Dopamine is one of the major neurotransmitters found in neurons of the SN. It has been estimated that about 90% neurons in the SNC and SNL are dopaminergic (Swanson, 1982). González-Hernández and Rodríguez (2000) also reported that nearly 20% of the total population of dopamine neurons in the VTA/SN are located in the SNR.

The VTA was classically described by Tsai (1925a, b) from Golgi and Nissl preparations. The VTA lies in the midline on the floor of the midbrain and contains a large number of dopamine neurons (more than 80%) known as the A10 dopamine cell group (Swanson, 1982) interspersed with GABA neurons (Oades and Halliday, 1987; Fallon and Loughlin, 1995). The dopamine neurons in the lateral part of the VTA merge with dopamine neurons of the SNC without a clear boundary between the two structures (see Fig. 1). Furthermore, it is widely agreed that the VTA is subdivided into several distinct cytoarchitectural regions such as parabrachial pigmented nucleus, paranigral nucleus, rostral linear nucleus and caudal linear nucleus, and interfasicular nucleus (Dahlstrom and

Fuxe, 1964; Palkovits and Jacobowitz, 1974; Phillipson, 1979b; Oades and Halliday, 1987; Fallon and Loughlin, 1995; González-Hernández and Rodríguez, 2000). The subregions of the VTA innervate different regions of the forebrain and thalamus (Oades and Halliday, 1987). However, there is considerable overlap in these innervation patterns and the functional significance of the different subdivisions of the VTA remains unknown.

1.3.2 Neurotransmitters in the Ventral Midbrain

1.3.2.1 Dopamine

It was recognized as early as the 1950's that dopamine may play an important role in neurotransmission when several investigations demonstrated that dopamine was present in the brain and that it was especially concentrated in the basal ganglia (Weil-Malherbe and Bone, 1957; Montagu, 1957; Carlsson *et al.*, 1958; Carlsson, 1959; Bertler and Rosengren, 1959a,b). In the 1960's, Swedish scientists described the presence of the major ascending dopamine systems that originate from dopamine neurons in the VTA and SN (Carlsson *et al.*, 1962; Dahlstrom and Fuxe, 1964; Fuxe, 1965; Anden *et al.*, 1966; Fuxe *et al.*, 1970; Ungerstedt, 1971). Since those early studies, numerous other studies have been done to map the central dopamine neuronal system using histofluorescence techniques and tyrosine hydroxylase (TH)

immunohistochemistry (Dahlstrom and Fuxe, 1964; Hokfelt *et al.*, 1974, 1976, 1980; Halasz *et al.*, 1977; Fuxe *et al.*, 1978; Moore and Bloom, 1978; Lindvall, and Bjorklund, 1978; Lindvall, and Bjorklund, 1983). Morphologically, there are four distinct ascending pathways (Fuxe, 1985; Oades and Halliday, 1987): 1) the nigrostriatal system, which originates from the SNC and projects to the dorsal striatum; 2) the mesolimbic system, which originates from the VTA and sends projection to the ventral striatum; 3) the mesolimbic-cortical system, which originates from the VTA and innervates the septum, amygdala and limbic cortex; 4) the mesothalamic system, which originates mainly from the medial VTA and activates medial thalamus associated with the limbic system (Troiano and Siegel, 1978; Phillipson, 1979a; Phillipson and Griffith, 1980; Tork *et al.*, 1984). These ascending dopamine neurons project to their targets through two major fiber systems, the medial forebrain bundle which contains dopamine fibers that innervate the striatum and limbic system, and the periventricular fiber system which innervates the thalamus region.

1.3.2.2 GABA

The other major type of neuron in the VTA and the SN is the GABA neuron, which forms a population of interneurons and projection neurons. GABA neurons in the SN are densely concentrated in the SNR and are distributed

sparsely in the SNL and SNC. GABA neurons in the SNR project to the thalamus, superior colliculus and pedunculopontine nucleus (Dahlstrom and fuxe, 1964; Kilpatrick *et al.*, 1980; Childs and Gale, 1983; Williams and Faull, 1985; Chiodo, 1988; Hedreen and DeLong, 1991; Parent and Hazrati, 1995). The SNR and the globus pallidus share morphological and chemical similarities (DeLong and Georgopoulos, 1979; Afifi, 1993), and serve as major output structures of the basal ganglia. Both structures project massively to specific thalamic relay nuclei, namely the ventral anterior and ventral lateral nuclei, which in turn project to the cerebral cortex (Alexander and Crutcher, 1990; Parent and Hazrati, 1995).

The VTA is composed of approximately 15-20% neurons that are non-dopaminergic (Mugnaini and Oertel, 1985, Kalivas, 1993; Fallon and Loghlin, 1995). These neurons are in close proximity to dopamine neurons and are believed to be GABA interneurons (Nagai et al., 1983; Otterson and Storm-Mathisen, 1984; Mugnaini and Oertel, 1985, Kalivas, 1993). However, it has also been demonstrated that a population of VTA neurons retrogradely labeled from the nucleus accumbens and the prefrontal cortex contains GABA (Van Bockstaele and Pickel, 1995; Carr and Sesack, 2000). The GABA projection neurons may form a portion of the mesolimbic-cortical pathway and play a role in regulating the activity of dopamine neurons implicated in behavioral reinforcement (Steffensen et al., 1998, 2001). In other words, the VTA GABA neurons are not only local inhibitory neurons but also projection neurons that

send axons to the cortex and nucleus accumbens, and potentially other areas of the brain.

1.3.2.3 Neuropeptides

Despite the fact that dopamine and GABA are found in the majority of neurons of the VTA and SN, several neuropeptides have also been localized within neurons of the ventral midbrain. Neurons immunoreactive for the neuropeptides neurotensin and cholecystokinin are also immunoreactive for TH, a marker for dopamine neurons in the midbrain (Seroogy et al., 1988). Neurons found to contain neurotensin or cholecystokinin are always found to be dopaminergic, which means that neurons of the VTA/SN never use cholecystokinin or neurotensin exclusively as neurotransmitter (Seroogy et al., 1988). The physiological implication of co-localization of peptides with dopamine remains somewhat controversial. Nevertheless, neuropeptides are likely to be co-released with dopamine and probably function in the regulation of dopamine release or act on neurotensin or cholecystokinin receptors in terminal fields of dopamine neurons (Seroogy et al., 1988).

1.3.2.4 Acetylcholine

In Mesulam's comprehensive mapping of acetylcholine neurons in the brain, there was no mention of cholinergic neurons in the VTA and SN (Mesulam *et al.*, 1984). On the other hand, using choline acetyltransferase (ChAT) immunohistochemistry, several studies have shown the existence of a small number of cholinergic neurons in the caudal SN (Sofroniew *et al.*, 1985; Woolf and Butcher, 1985; Gould and Butcher, 1986; Moriizumi *et al.*, 1991) as well as the dorsal and lateral parts of the middle caudal levels of the SNR, particularly in the borders of the SNC, SNR and SNL (Gould and Butcher, 1986; Martinez-Murillo *et al.*, 1989). These weakly stained ChAT-positive neurons are thought to be projection neurons that innervate the superior colliculus. It has also been suggested that cholinergic neurons may play an important role in the regulation of forebrain activation and locomotion (Gould and Butcher, 1986; Martinez-Murillo *et al.*, 1989; Moriizumi *et al.*, 1991).

1.3.3 Descending Projections from the VTA/SN

In addition to these well-known ascending dopaminergic and GABAergic projections of the VTA/SN, there is some evidence for descending projections to the locus coeruleus, lateral parabrachial nucleus, cerebellum and the

periaqueductal gray (Beckstead *et al.*, 1979; Simon *et al.*, 1979; *Ouimet et al.*, 1984; Fuxe *et al.*, 1985). It must be emphasized that the chemical nature of descending projections from the VTA and SN is not known. However, dopamine fibers and terminals have been demonstrated in those brainstem regions with immunohistochemical techniques (Kitahama *et al.*, 2000). Dopamine receptors have also been demonstrated in these brain regions (Kirouac and Ganguly, 1992). The chemical nature of descending projections from the VTA and SN to the brainstem would have to be examined using retrograde tract-tracing techniques combined with immunohistochemistry for neurotransmitters such as dopamine. Therefore, the details of these descending projections remain largely unexplored.

1.4 Role of the Ventral Midbrain in Cardiovascular Regulation

1.4.1 Ventral Tegmental Area and Substantia Nigra (VTA/SN)

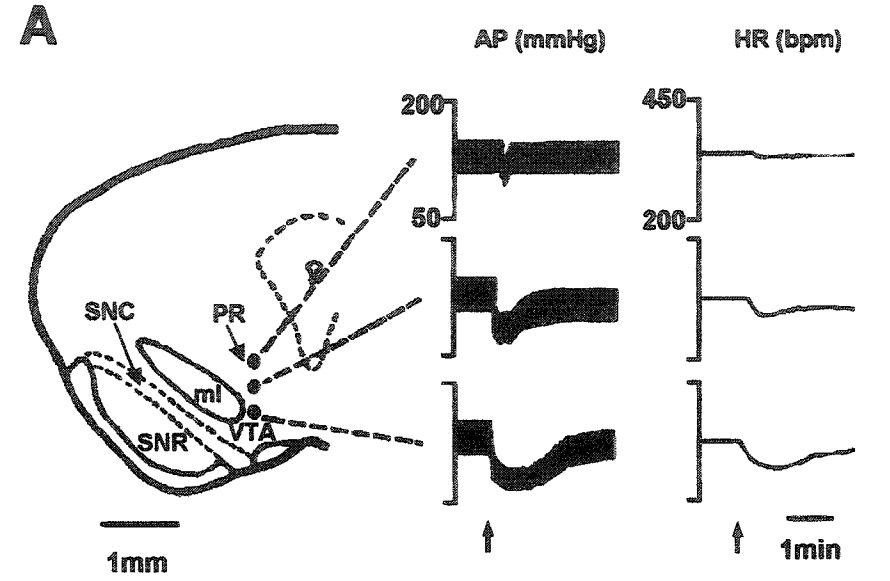
As previously described, dopamine pathways are generally thought to function in the regulation of motor and behavioral responses mediated by neuronal mechanisms in the forebrain (Kohler *et al.*, 1985; Alexander *et al.*, 1990). However, recent experimental evidence suggests that the mesencephalic dopamine system may play an important role in the regulation of the

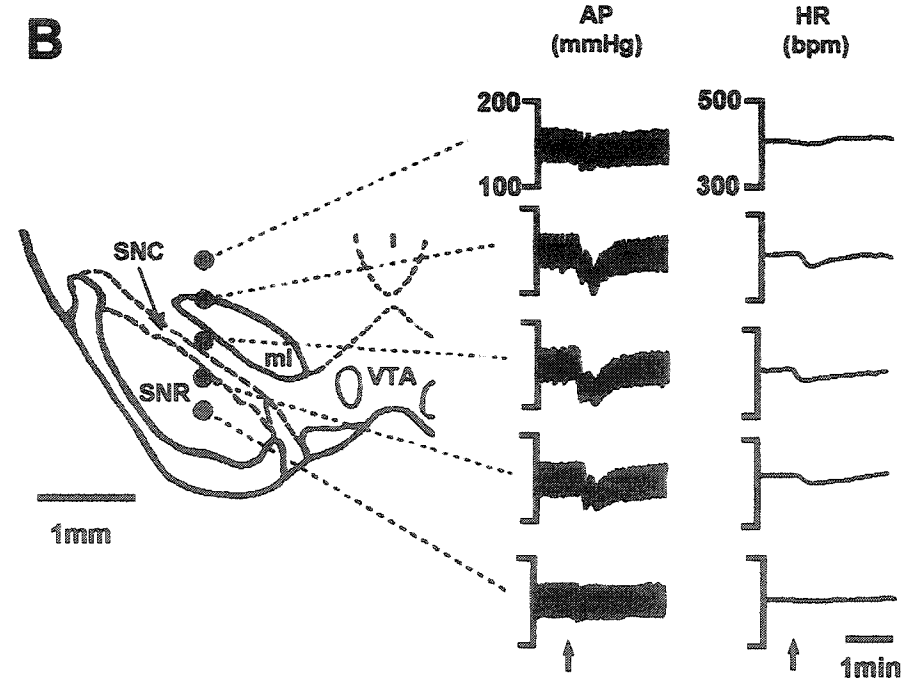
cardiovascular system. For example, some dopamine neurons in the VTA were found to respond to baroreceptor activation produced by systemic injections of the adrenoreceptor agonist phenylephrine (Kirouac and Ciriello, 1997a). Stimulation of the VTA electrically or with microinjection of the excitatory amino acid L-glutamate has been shown to elicit variable changes in arterial pressure (AP) and heart rate (HR) in the rat and rabbit (Dahlstrom and Fuxe, 1964; Oades and Halliday, 1987; Cornish *et al.*, 1997; Stotz-Potter and Benarroch, 1998). Similarly, electrical stimulation of the SN in the cat (Beitz, 1982; Angyan, 1989; Bouthenet *et al.*, 1991) or the rat (Otake *et al.*, 1994) elicited increase in AP and HR. In addition, chemical stimulation of the SN with relatively high concentrations and large volumes of L-glutamate or kainic acid was reported to elicit responses in the rat similar to those elicited by electrical stimulation (Otake *et al.*, 1994). It was suggested that the cardiovascular responses to stimulation of the VTA and SN were mediated by dopamine projections because these responses were blocked by the peripheral and central administration of a dopamine antagonist (Dahlstrom and Fuxe, 1964; Otake *et al.*, 1994; Cornish *et al.*, 1997).

The location of the neurons in the ventral midbrain that may be responsible for these cardiovascular responses was not clearly defined in these studies for two reasons. First, the large microinjections of neuroactive substances used to produce cardiovascular responses (Dahlstrom and Fuxe, 1964; Otake *et al.*, 1994; Cornish *et al.*, 1997; Stotz-Potter and Benarroch, 1998)

may have stimulated neurons outside the VTA or SN. Second, electrical stimulation used to elicit the cardiovascular responses in the VTA/SN may have activated fibers that course through this region (Domesick, 1988; Fallon, 1988; Fallon and Loughlin, 1995). Kirouac and Ciriello (1997b) systematically explored the ventral midbrain for cardiovascular-responsive sites by injecting small volumes of the excitatory amino acid L-glutamate, which has been shown to selectively excite neuronal cell bodies but not fibers of passage (Goodchild *et al.*, 1982). This study demonstrated that L-glutamate stimulation of the VTA and SN elicited decreases in mean AP (MAP) that were often accompanied by decreases in HR (see Fig.2 for example). Systemic administration of the muscarinic receptor antagonist atropine, which blocks the parasympathetic stimulation of the heart, did not alter the magnitude of the cardiovascular responses. Administration of the nicotinic receptor antagonist hexamethonium bromide, which blocks the sympathetic stimulation of the vasculature and heart, abolished the MAP responses and significantly attenuated the HR responses. This suggests that the cardiovascular responses elicited from the VTA/SN are mediated by inhibition of the sympathetic activity to the vasculature and the heart. Stimulation of the VTA and SNC consistently elicited the most marked cardiovascular responses, while regions immediately adjacent to VTA and SNC had smaller effects. As shown in Figure 2, the largest depressor responses were elicited when the microinjections of L-glutamate were in the region where dopamine neurons are located in the VTA and SNC (Kirouac and Ciriello, 1997b).

Fig. 2. Representative micropipette tract through the region of ventral mesencephalon involving the ventral tegmental area (A) and the substantia nigra (B) showing the arterial pressure (AP) and heart rate (HR) responses elicited during microinjection of L-glutamate at different dorsoventral sites (filled circles). Note that the magnitude of the cardiovascular depressor responses elicited from stimulation of the prerubral field increases as the micropipette tract approaches the ventral tegmental area, at which point the largest cardiovascular responses are elicited (A). The largest cardiovascular responses in the ventral mesencephalon were elicited from the region of the compact part of the substantia nigra, whereas no responses were elicited by stimulation of reticular part of the substantia nigra or reticular formation of dorsal tegmentum (B). Arrows, time of L-glutamate injection. VTA, ventral tegmental area; SNC, compact part of the substantia nigra; SNR, reticular part of the substantia nigra; PR, prerubral field; ml, medial lemniscus. Figures are produced from Kirouac and Ciriello (1997).


A
AP (mmHg)
HR (bpm)
200
50
450
200
SNC
PR
ml
VTA
SNR
1mm
1min


B
AP
(mmHg)
HR
(bpm)
200
100
500
300
SNC
ml
VTA
SNR
1mm
1min

Moreover the depressor responses elicited by stimulation of the VTA appear to involve dopamine D2 receptors because pretreatment with the D2 dopamine antagonist raclopride attenuated the depressor responses from stimulation of the VTA (Kirouac and Ciriello, 1997b). Therefore, dopamine is a possible candidate for mediating the cardiovascular responses produced by the VTA/SN (Oades and Halliday, 1987).

It is known that the VTA/SN projects to many forebrain regions that have been implicated in the regulation of the cardiovascular system (Oades and Halliday, 1987). Efferent fibers from the VTA/SN travel through the medial forebrain bundle and the periventricular fiber system to innervate the forebrain and thalamus. The local anesthetic lidocaine was microinjected in the ascending fiber bundles to chemically block nerve impulse traffic to determine the pathway mediating the cardiovascular depressor responses elicited from stimulation of the VTA/SN. Bilateral microinjection of lidocaine in either the medial forebrain bundle or the periventricular fiber system did not attenuate the depressor responses from stimulation of the VTA/SN, which indicated that the VTA/SN depressor responses were not mediated by ascending projections to the forebrain. In an attempt to determine the relay for the VTA/SN cardiovascular responses, Kirouac and Pittman (2000) injected the sensitive anterograde tracer biotinylated dextran amine in the VTA/SN. The brainstem was then examined to see if the VTA/SN projected to brainstem areas involved in the control of the autonomic nervous system. A dense bilateral projection was found to innervate the PAGvl and the

lateral wing of the DR (Kirouac and Pittman, 2000), regions that have been strongly implicated in cardiovascular control (Bandler *et al.*, 1991; Carrive and Bandler, 1991; Bandler and Shipley, 1994; Dampney, 1994; Sun, 1995). Subsequent experiments showed that blockade of neuronal transmission with lidocaine or blockade of synaptic transmission with cobalt chloride in the PAGvl/DR region attenuated the cardiovascular depressor responses produced by stimulation of the VTA/SN (Kirouac and Pittman, 2000). This series of experiments indicated that the depressor responses produced by stimulation of the VTA/SN were mediated by a relay to the PAGvl/DR. A low to moderate number of dopamine D2 receptors have also been demonstrated in the PAG using the sensitive in situ hybridization method (Weiner *et al.*, 1991). However, there is no evidence in the literature that dopaminergic neurons in the VTA project to the PAG or that exogenous dopamine in the PAG produces cardiovascular responses.

1.4.2 Periaqueductal Gray and Dorsal Raphe Nucleus (PAG/DR)

1.4.2.1 Midbrain Mechanisms in Analgesia and Cardiovascular Regulation

The periaqueductal gray (PAG) is a midbrain structure that is located around the central canal from the posterior commissure to the rostral locus coeruleus. Considerable evidence supports an important role for the PAG in the

regulation of defensive behaviors, autonomic function and pain transmission (Lovick, 1993; Bandler and Shipley, 1994; Behbehani, 1995). On the basis of the results of chemical and electrical stimulation studies, Bandler and colleagues proposed that the PAG consists of functionally distinct longitudinal columns that mediate specific physiological responses (Carrive, 1991; Lovick, 1993; Bandler and Shipley, 1994; Behbehani, 1995; Bandler *et al.*, 2000). For example, stimulation of the dorsal lateral column of the PAG with excitatory amino acids produces attack responses whereas stimulation of the lateral column of the PAG produces flight behavior. Hypertension, tachycardia and a non-opioid analgesia are produced from stimulation of the dorsolateral and lateral PAG. In contrast, stimulation of the ventrolateral column of the PAG (PAGvl) produces freezing behavior or hyporeactivity with hypotension, bradycardia and opioid mediated analgesia. The dorsal raphe nucleus (DR), which is composed of serotonin neurons, is in part embedded in the caudal portion of the PAGvl. The DR has also been implicated in nociceptive and cardiovascular regulation (Robinson *et al.*, 1986; Connor and Higgins, 1990; Wang and Nakai, 1994) and is believed to play an important role in regulating the neural circuitry in the PAG mediating emotional reactions to fear and pain (Lovick, 1993).

The PAG receives afferents from a large number of brain regions implicated in the regulation of emotions and the physiological responses associated with emotional reactions (Bandler and Shipley, 1994; Behbehani, 1995). The brain regions with the most prominent projections to the PAG and DR

include the limbic cortices, hypothalamus, amygdala, dorsal horn of the spinal cord and variety of brainstem regions associated with the regulation of the autonomic and somatic nervous system (Sakai *et al.*, 1977; Marchand and Hagino, 1983; Beitz, 1982; Peyron *et al.*, 1995; Floyd *et al.*, 2000). Recent experiments by Kirouac and Pittman (2000) showed that the PAG and DR were innervated by the VTA and SN, two areas that are also implicated in the regulation of behavioral and motor functions.

1.4.2.2 PAG Mediated Hypotensive Responses

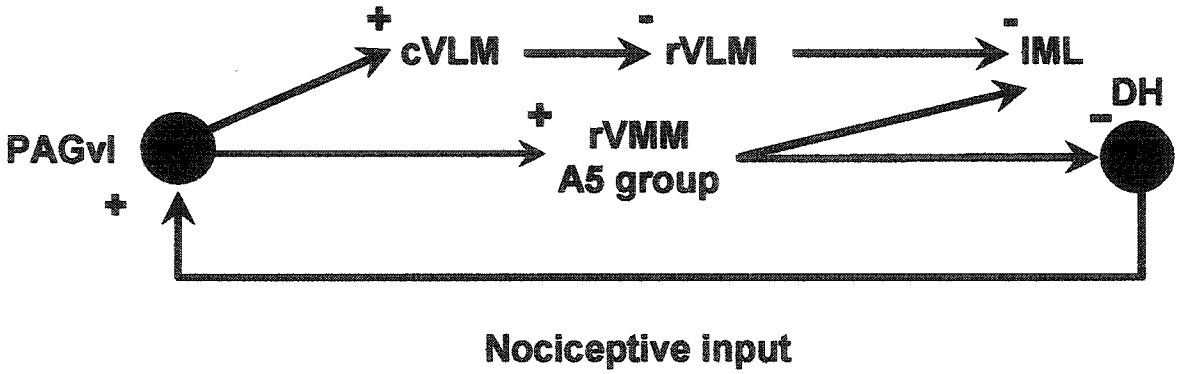
A large amount of literature has accumulated on the role of the PAG in regulating emotional expression and cardiovascular function (Bandler *et al.*, 1991; Carrive and Bandler, 1991; Bandler and Shipley, 1994; Dampney, 1994; Behbehani, 1995; Sun, 1995). Studies using local microinjections of excitatory amino acids to selectively activate neurons in the PAG have revealed two functional longitudinal columns of cells that produce distinctive cardiovascular responses (Bandler and Shipley, 1994) as mentioned above: stimulation of the lateral PAG (PAGl) produces hypertension and tachycardia while stimulation of the ventrolateral PAG (PAGvl) produces hypotension and bradycardia (Lovick, 1993; Bandler and Shipley, 1994). Acute hypotensive episodes were found to increase the expression of c-*fos* in the PAGvl, indicating that neurons in the

PAGvl are activated by unloading of the baroreceptors (Li and Dampney, 1995; Murphy *et al.*, 1995; Tassoreli and Joseph, 1995). It is interesting to note that the hypotension and bradycardia produced by stimulation of the PAGvl are also seen in response to severe blood loss and pain (Schadt and Ludbrook, 1991; Bruehl *et al.*, 1999). Indeed, the PAGvl stands out as a region of the brain where neurons are excited by deep somatic and visceral nociceptive inputs and may be involved in the production of cardiovascular responses to pain (Bandler and Shipley, 1994). In summary, previous experiments suggest that the PAGvl is a critical brain region in which deep pain and haemorrhage inputs may be integrated to produce hypotension and bradycardia.

1.4.3 Descending Connections from the PAGvl Mediating Hypotension and Antinociception

Figure 3 shows a schematized summary of the circuitry involved in PAGvl induced antinociception and hypotension. PAGvl induced antinociception and hypotension are mediated by a direct excitatory projection to rostral ventromedial medulla, caudal ventrolateral medulla and the A5 noradrenergic cell group located in the pons (Basbaum and Fields, 1984; Fields *et al.*, 1991; Behbehani, 1995). The rostral ventrolateral medulla and A5 cell group project directly to the

Fig.3. Schematized summary of the circuitry involved in ventrolateral periaqueductal gray (PAGvl) induced antinociception and hypotension. PAGvl induced antinociception and hypotension are mediated by a direct excitatory projection to rostral ventromedial medulla, caudal ventrolateral medulla and the A5 noradrenergic cell group located in the pons. The rostral ventrolateral medulla and A5 cell group project directly to the dorsal horn of the spinal cord where transmission of nociceptive information is inhibited. The rostral ventrolateral medulla and the A5 cell group also project to preganglionic sympathetic neurons in the intermediolateral cell column and activation of these descending pathways can produce vasodilation in the circulation by inhibiting preganglionic sympathetic motor neurons. Neurons in the caudal ventrolateral medulla in turn innervate the intermediolateral cell column. The PAGvl also receives direct input from nociceptive neurons and this input serves as a feedback circuit, which when activated, produces descending inhibition of nociceptive transmission at the level of the spinal cord and cardiovascular responses. PAGvl, ventrolateral periaqueductal gray; rVLM, rostral ventrolateral medulla; cVLM, caudal ventrolateral medulla; IML, intermediolateral cell column; DH, dorsal horn of the spinal cord.


+
cVLM
-
rVLM
-
IML
DH
-
+
rVMM
A5 group
PAGvl
+
Nociceptive input

dorsal horn of the spinal cord where transmission of nociceptive information is inhibited (Basbaum and Fields, 1984; Fields *et al.*, 1991; Behbehani, 1995). The rostral ventrolateral medulla and the A5 cell group also project to preganglionic sympathetic neurons in the intermediolateral cell column and activation of these descending pathways can produce vasodilation in the circulation by inhibiting preganglionic sympathetic motor neurons (Dampney, 1994; Sun, 1995). Neurons in the caudal ventrolateral medulla in turn innervate the intermediolateral cell column. The PAGvl also receives direct input from nociceptive neurons and this input serves as a feedback circuit, which when activated, produces descending inhibition of nociceptive transmission at the level of the spinal cord and cardiovascular responses (Bandler and Shipley, 1994). As shown schematically in Figure 3, sensory neurons in the dorsal horn of the spinal cord and trigeminal sensory nucleus as well as neurons in the central gray of the spinal cord project directly to the PAGvl providing an anatomical substrate for the transmission of nociceptive information to the PAGvl (Keay *et al.*, 1997). The PAGvl also receives a strong input from the nucleus of the solitary tract and parabrachial nucleus, which relay visceral nociceptive and baroreceptive information to the brain (Clement *et al.*, 1996).

An anatomical projection between the VTA/SN and the PAGvl/DR is of potential importance because of the reports indicating that the VTA/SN plays a significant role in modulating pain and arterial blood pressure, two functions that are regulated by the PAGvl/DR. Several studies have also implicated the VTA

and SN in cardiovascular regulation. For example, stimulation of the VTA and the SN with excitatory amino acids produces cardiovascular depressor responses in anesthetized rats (Kirouac and Ciriello, 1997b; Zhang *et al.*, 1997; Kirouac and Pittman, 2000), which appear to be mediated by a direct projection to the PAGvl/DR (Kirouac and Pittman, 2000). While the hypothesis that antinociception elicited by activation of neurons in the VTA/SN may be mediated by a projection to the PAG or DR has not been tested, it is plausible considering the existence of a moderately dense projection between the VTA/SN and PAG as well as the role the PAG plays in the modulation of pain transmission.

1.5 Hypothesis

In a recent paper, Kirouac & Pittman (2000) demonstrated a functional link between the VTA/SN and the PAGvl/DR region by showing that the cardiovascular depressor responses elicited by stimulation of the VTA/SN region were attenuated by blocking impulse transmission to the PAGvl/DR region. Previous anatomical studies have demonstrated a connection between the VTA/SN and the PAGvl/DR (Sakai *et al.*, 1977; Beckstead *et al.*, 1979; Simon *et al.*, 1979; Marchand and Hagino, 1983; Kalen *et al.*, 1988; Beitz, 1982; Peyron *et al.*, 1995). However, a detailed description of the projection with consideration

given to the anatomical and functional columns of the PAG was not done in these studies. Kirouac and Pittman (2000) using the sensitive anterograde tracer biotinylated dextran amine confirmed that the PAGvl/DR received a dense projection from the VTA/SN. However, the location of the neurons in the VTA/SN that project to the PAGvl/DR could not be discerned by injections of biotinylated dextran amine that often involved several anatomically distinct subregions of the VTA/SN (Kirouac and Pittman, 2000). Despite the potential importance of a connection between these midbrain regions, the connection between the VTA/SN and the PAGvl/DR has not been the subject of detailed anatomical studies.

The experiments in this thesis are based on the hypothesis that a dopaminergic projection from the VTA and SN to the PAGvl/DR mediates the cardiovascular depressor responses produced by stimulation of the VTA/SN. The hypothesis is derived from the following observations:

1. the most effective sites for eliciting cardiovascular depressor responses are in the VTA and SN where dopamine neurons are located (Kirouac and Ciriello, 1997b).
2. blocking of dopamine D2 receptors with an intravenous injection of a D2 antagonist attenuates the cardiovascular depressor responses (Kirouac and Ciriello, 1997b).
3. dopamine neurons form the majority of projection neurons in the VTA and SNC (Fuxe *et al.*, 1985; Follan and Loughlin, 1995).

1.6 Objectives

The aims of the present study were:

1. to describe the projections of the VTA and SN to functionally distinct regions of the PAGvl including the DR.
2. to examine if other subdivisions of the PAG also receive innervation from the VTA/SN.
3. to determine if the neurotransmitter content of the projection is dopamine or GABA.
4. to determine if the cardiovascular depressor responses elicited by stimulation of the VTA/SN are mediated by dopaminergic or GABAergic projection neurons.

Chapter 2

Methods and Materials

2.1 Animals

The experimental protocols for this research were according to the guidelines set by the Canadian Council on Animal Care and the research protocols were approved by the Internal Animal Care Committee of Memorial University of Newfoundland.

Male adult Sprague-Dawley rats weighing 275 to 450 g were used for the experiments. The animals were housed in the animal care unit and kept on a 12 hours light and dark cycle.

2.2 Tract-tracing Experiments

A major aim of the work presented in this thesis was to describe the connections between the VTA/SN and the PAG region. This was done by injecting the retrograde tracer cholera toxin B (CTb) into various regions of the PAG of the rat. Following a sufficient period of time for the CTb to be taken up by fiber terminals and retrogradely transported to neurons in the VTA/SN, the rat was perfused with fixative, brain tissue was cut and processed for CTb immunohisto-chemistry to visualize the location of VTA/SN neurons projecting to the PAG. Another major aim of this thesis was to determine the neurotransmitter used by the VTA/SN projection to the PAG region. Therefore, a second series of double-labeling experiments were done using immunohistochemistry or immunofluo-rescence combined with retrograde tract tracing with CTb or FluroGold (FG). Injections of CTb and FG were done in the PAG as before and tissue was immunoreacted for tyrosine hydroxylase (TH) or glutamic acid decarboxylase (GAD) to visualize the tracer and the putative neurotransmitter (dopamine or GABA). The following tract-tracing experiments were done:

1. CTb tract-tracing alone (reacted immunohistochemically with DAB).
2. CTb combined with TH and GAD immunohistochemistry (DAB reacted).
3. FG combined with GAD immunofluoresescence (fluorescence).
4. CTb combined with TH and GAD immunofluorescence (fluorescence).

2.2.1 Injection of Tracers

Rats were anesthetized with equithesin (0.3 ml/100g body weight, i.p.) and given supplementary doses (0.15 ml/100g body weight, i.p.) as needed during the surgical procedure. Rats were placed in a Kopf stereotaxic frame with the incisor bar set at 3.3 mm below the interaural line (Paxions and Watson, 1986). The body temperature was monitored and maintained at 35-37 °C with a heating pad. A burr hole of approximately 1 mm in diameter was made for the injection. Glass micropipettes with fine tip diameters were used to iontophoretically inject the retrograde tracers in the PAG region. Glass micropipettes (1.00 mm i.d., 1.50 mm o.d.; Garner Glass Company, Claremont, CA) were heated and pulled with a vertical pipette puller (David Kopf Instruments, Tujunga, CA), and tips were broken to a certain diameter (15-25 μm), which were verified with a microscope fitted with a micrometer.

The coordinates used for injection of tracers in the caudal PAGvl were 1.5 to 2.0 mm anterior to the interaural line, 1.0 mm lateral to the midline, and 5.0 to 5.5 mm below the dorsal surface of the brain. Control injections were also done outside the PAGvl by making adjustments to the above coordinates. The scalp incision was sutured and rats were returned to individual cages for recovery during which they were closely monitored for signs of pain or distress.

2.2.1.1 Cholera toxin B (CTb) Injection

Fine glass pipettes (15-25 µm tip diameter) were used to inject CTb. The low salt version of CTb was reconstituted with distilled water to make a solution of 0.5% in 0.1 M sodium phosphate buffer (List Biological Laboratory, Campbell, CA). The iontophoretic injections of CTb were done by applying a 3 to 5 µA positive current (200 ms pulses at 2 Hz for 10 to 20 min) through a chlorinated silver wire placed in the pipette.

2.2.1.2 FluroGold (hydroxystilbamidine methanesulfonate) Injection

Glass micropipettes (20-25 µm tip diameter) were used to inject FluroGold (FG). A 0.5-1% solution of FG (Molecular Probes, Inc., Eugene, OR, USA) was made by dissolving 10 mg of FG in 1-2 ml of 0.1 M cacodylate buffer. The iontophoretic injections were done by applying a 4 to 5 µA positive current (200 ms pulses at 2 Hz for 20 to 30 min) or 2 µA positive current (7 seconds on/off for 15 min) through a chlorinated silver wire placed in the pipette. It is more difficult to produce dense injections of FG by iontophoresis than it is for CTb (personal experience). This problem was addressed by doing injections with a 7s on/off protocol, which seems to give injections that were denser than with the 200 ms

pulses protocol. The on/off current application prevents the pipette tip from being blocked during the application of a direct electrical current.

2.2.2 Colchicine Treatment for GAD Immunohistochemistry

To increase the detectable levels of GAD in the cell body of neurons, rats that had previously received CTb injections in the PAG/DR were treated with colchicine 1 to 2 days before perfusion with fixative. Rats were anesthetized with equithesin (0.3 ml/100g body weight, i.p.) and placed in a stereotaxic frame as described in the previous section. A solution of 50 to 100 μg of colchicine (6 μl, Sigma) was injected into the lateral ventricles. The coordinates for this injection was 1.3 mm posterior to bregma, 0.8 mm lateral to the midline, and 3.0 to 3.5 mm below the dorsal surface of the brain. The colchicine solution was microinjected by the application of pressurized nitrogen pulses controlled by a pneumatic pump (Medical Systems, Great Neck, NY) using a micropipette with the total volume of 20 μl (Division American Hospital Supply Corporation, Miami, Fla) and the tip diameter around 75 μm. The scalp incision was sutured and rats were returned to individual cages for recovery.

Colchicine is a well-known chemical that has been used to interrupt axonal transport. After injection of colchicine, newly synthesized proteins stay in the cell body where they are synthesized instead of being transported to the axonal

terminals. Therefore, with the treatment of colchicine, GAD proteins will accumulate in the cell body, which then could be detected more easily (Ribak *et al.*, 1978).

2.3 Perfusion and Tissue Processing

After a survival period of 7 to 14 days, rats were deeply anesthetized and perfused with 150 ml of saline containing 0.5% heparin followed by 500 ml of ice-cold fixative composed of 4% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed and postfixed in the same fixative at room temperature for 1 hr before being transferred to a 20% sucrose solution and stored at 4°C overnight for cryoprotection.

Coronal sections of the midbrain were cut at 25 to 50 µm using a freezing microtome (Leitz Wetzlar) or a cryostat (Ultrapro 5000, Vibratome, St. Louis, MO) and collected in PBS prior to the immunohistochemical reaction. Some brain tissue processed for CTb immunohistochemistry was cut at 50-75 µm using a vibratome (Vibratome, St. Louis, MO). All sections processed for immunohisto-chemical detections of CTb or putative neurotransmitters were reacted on free-floating sections. All antibodies were diluted in 0.1 M PBS containing normal

donkey serum (5 to 10%), 0.01-0.3% Triton X-100 and 0.1% sodium azide. Once all chemical reactions were complete, the sections were mounted on gelatin-coated slides, dried at room temperature, and coverslips were applied using Fluka DPX mountant.

2.3.1 CTb Immunohistochemistry (DAB)

Following rinses in PBS (3 x 10 min), sections were incubated in goat anti-CTb (1:10,000 to 40,000; catalog number 703, List Biological Laboratory, Campbell, CA) at 4°C for 1 to 2 days. Sections were rinsed and incubated in biotinylated donkey anti-goat (1:500, catalog number 705-065-147, Jackson ImmunoResearch, West Grove, PA) for 2 hrs followed by rinses and 1 hr incubation at room temperature in a avidin-biotin complex according to kit directions (Elite ABC Kit, Vector Laboratories, Burlingame, CA). This was followed by three rinses, incubation in diaminobenzidine for 2.5 to 10 min according to kit directions (DAB peroxidase substrate kit, Vector Laboratories, Burlingame, CA). After several rinses, sections of the PAG were mounted on gelatin-coated slides.

2.3.2 Double-labeling Experiments

2.3.2.1 Antibodies for Neurotransmitter Identification

Two TH antibodies were used for these experiments. The rabbit anti-tyrosine hydroxylase antibody (catalog number AHP368, Serotec, Raleigh, NC) was a polyclonal antibody that gave dark cell body staining at a high concentration (1:200 to 1:500). The monoclonal mouse anti-tyrosine hydroxylase (catalog number 12K4863, Sigma), in contrast, was used in a very low concentration (1:40,000), which resulted in dark cell body labeling with a much lower background staining. Most of the immunofluorescent experiments were with the monoclonal anti-TH antibody.

There were three different GAD antibodies that were used for the experiments in this thesis: rabbit anti-glutamate decarboxylase (GAD) polyclonal antibody (catalog number AB108, Chemicon International, Temecula, CA); rabbit anti-glutamate decarboxylase$_{65/67}$ (GAD$_{65/67}$) polyclonal antibody (catalog number AB1511, Chemicon International, Temecula, CA), which recognizes both isoforms of GAD (GAD$_{65/67}$) thereby labeling GAD in both fiber terminals and cell bodies (Esclapez et al., 1994; Dirkx et al., 1995); rabbit anti-glutamate decarboxylase67 (GAD67) polyclonal antibody (catalog number 228485, Alpha Diagnostic, San Antonio, TX, USA). In addition, several clones of a new

monoclonal GAD antibody (samples received from Chemicon International) were also used. Discussion on the use of these various GAD antibodies is presented in the Results section.

2.3.2.2 Double-labeling Experiments for Immunohistochemistry (DAB)

Sections were first reacted for CTb as described in section 2.3.1, but instead of being mounted on slides, the sections of the VTA/SN were incubated in rabbit anti-TH (1:500; Serotec, Raleigh, NC) or rabbit anti-GAD (1:500; Chemicon International, Temecula, CA) at 4°C for 1 to 2 days. Sections were rinsed and incubated in biotinylated donkey anti-rabbit (1:500; catalog number 711-065-152, Jackson ImmunoResearch, West Grove, PA) for 2 hrs. After several rinses and 1 hr incubation in an avidin-biotin complex (Elite ABC Kit, Vector Laboratories, Burlingame, CA) at room temperature, sections were rinsed and incubated in diaminobenzidine without Nickel solution for 2.5 to 10 min (DAB peroxidase substrate kit, Vector Laboratories, Burlingame, CA). After rinses, sections were mounted and the slides were coverslipped.

2.3.2.3 Double-labeling Experiments for Immunofluorescence

FG Experiments Combined with GAD Immunofluorescence

Antibodies for GAD were diluted in 0.1 M PBS containing 10% normal Donkey serum. Free-floating sections were pre-incubated in the buffer for 30 to 60 min, and incubated in rabbit anti-glutamate decarboxylase (GAD; 1:500; catalog number AB1511, Chemicon International, Temecula, CA) at 4°C for 2 days. After rinses, brain sections were incubated in Cy_3 conjugated donkey anti-rabbit (1:500; Jackson ImmunoResearch, West Grove, PA) for 3 hours at room temperature. After a few more rinses, tissue sections were mounted on slides and coverslipped. FG is a substance that fluoresces under an ultraviolet (UV) filter and therefore no further procedure is needed to visualize FG labeled cells or injection sites.

CTb Experiments Combined with TH and GAD Immunofluorescence

The tissue for CTb and TH or GAD double labeling was reacted in a mixture of goat anti-CTb (1:10,000 to 40,000; List Biological Laboratories, Campbell, CA) and monoclonal mouse anti-TH (1:40,000, Sigma) or monoclonal mouse anti-GAD (1:500; Chemicon International, Temecula, CA) at 4°C for 1 to 2

days. This GAD antibody recognizes the 67K isoform of GAD that is preferable for labeling cell bodies (Esclapez et al., 1994; Dirkx et al., 1995). After rinses, brain sections were incubated in another mixture of antibodies containing Cy_2 conjugated donkey anti-goat (1:500; Jackson ImmunoResearch, West Grove, PA) and Cy_3 conjugated donkey anti-mouse (1:500; Jackson ImmunoResearch, West Grove, PA). After a few more rinses, tissue sections were mounted on slides and coverslipped.

Triton X-100 was used at a relatively low concentration of 0.01% in the blocking buffer for experiments to show GAD positive neurons because high concentrations of Triton X-100 results in the preferential labeling of GAD in fiber terminals. A low concentration of Triton X-100 is necessary for CTb antibodies to penetrate sufficiently (unpublished observations). At a concentration of 0.01% Triton X-100, both GAD and CTb immunofluorescence could be carried out on the same tissue sections (personal experience).

Control experiments were done by removing the primary antibody against CTb, TH, and GAD, which resulted in the absence of the labeling observed when the immunochemical reactions were done with the primary antibody present.

2.4 Analysis of CTb, TH and GAD Immunohistochemistry

Coronal sections from the caudal region of the PAG to the rostral region of the VTA/SN were examined using a light microscope. The location of the CTb injection site was defined and the distribution of retrogradely labeled cells was analyzed. In some representative cases, the location of CTb-labeled neurons that resulted from injections of CTb in the different regions of the PAG was drawn using a drawing tube. Double-labeled neurons were analyzed with an Olympus fluorescent microscope (BX51) equipped with appropriate filter cubes for Cy_2 (U-MNB2, Olympus), Cy_3 (U-MNG2, Olympus) or combination narrow green and blue filter (U-M51006ZZ, Olympus) combined with a blue/green excitation balancer (U-EXBABG, Olympus) to optimize the contrast under the single filter cube. The number of retrogradely labeled cells (CTb+) that are also labeled for TH (TH+) or GAD (GAD+) was counted to determine the contribution of the putative neurotransmitters to the innervation of the PAGvl/DR. Counts were done bilaterally on four representative sections that represented the rostrocaudal extent of the VTA and SN (approximate levels represented in Fig. 6B, 6C, 6D, 6E in the result section) in 4 rats that had CTb injections confined the PAGvl and wings of the DR. Counts were classified according to the approximate area in which the CTb+ neurons were found, including the VTA, SNC, SNR and SNL. Photomicrographs were produced using a digital camera (Spot RT Slicer,

Diagnostic Instruments, Sterling Heights, MI) and the images were imported into Adobe Photoshop 5.5 for contrast and color enhancement.

2.5 Physiological Experiments

Experiments were done on 10 male Sprague-Dawley rats (300–450 g) that were anesthetized using urethane (1.6 g/kg, i.p.). Polyethylene catheters (PE-50) were inserted into the femoral artery and vein for the recording of arterial pressure and the administration of drugs, respectively. Rectal temperature was monitored and maintained at 35–37°C with a heating pad. Experiments were done with rats placed in a stereotaxic frame with the nose bar adjusted 3.3 mm below the interaural line according to the stereotaxic atlas of Paxinos and Watson (1986).

Arterial blood pressure was recorded using a pressure transducer (MLT1050; ADInstruments, Mountain View, CA) connected to a bridge amplifier (ML110; ADInstruments). The electronic signal for arterial pressure was digitized using a PowerLab 4SP device and software (ADInstruments) and the data were captured and analyzed on a computer (Chart 4.0 data capture and analysis software; ADInstruments). Heart rate was calculated online from the pressure

pulse and the mean arterial pressure (MAP) was calculated as the diastolic pressure plus one-third of the pressure pulse.

Glass micropipettes with tip diameters of 30–50 µm containing a 0.01M glutamate (sodium salt, Sigma) dissolved in 0.1M PBS were lowered in the ventral midbrain on the right side (5.0–5.5 mm caudal to bregma, 0.8–1.3 mm lateral to the midline, and 6.5–8.5 ventral to the dura), and 50 nl of the glutamate solution were microinjected by the application of pressurized nitrogen pulses controlled by a pneumatic pump (Medical Systems, Great Neck, NY). The injected volumes were measured by the direct observation of the fluid meniscus in the micropipettes with an engineering microscope fitted with a micrometer (Titan, Buffalo, New York). Regions of the VTA/SN were stimulated with glutamate to locate sites that produced depressor responses of at least 15 mmHg. A minimal distance of 300 µm separated each injection site.

It was previously established that injection of the vehicle in the VTA/SN does not produce changes in arterial pressure or heart rate (Kirouac and Ciriello, 1997; Zhang *et al.*, 1997; Kirouac and Pittman, 2000). The cardiovascular depressor responses to glutamate microinjections into the VTA were retested after administration of the GABA blocking substance picrotoxin (2.5 nmol/500 nl; Sigma) in the region of the PAGvl/DR. Glass micropipettes (50 to 75 µm tip) were used to microinject picrotoxin with pressure pulses (8.0 mm posterior to bregma; 0.5 mm lateral to the midline; 5.0 mm ventral to the dura; angled 6° in the posterior direction). The following protocol was used:

1) a site in the VTA/SN that produced a depressor response of a minimum of 15 mmHg was identified;

2) 15 min later, picrotoxin was injected in the PAGvl/DR;

3) the VTA site was restimulated at 5, 30, and 60 min after the picrotoxin injection.

Only one experiment was done in each animal, which was followed by transcardial perfusion of 100 ml saline followed by 200 ml of 10% formalin solution. Sections of the brain were cut on a cryostat and stained with thionin to verify the placements of the micropipettes in the VTA/SN and the PAGvl/DR. The effects of administration of picrotoxin in the PAGvl/DR on the depressor responses were analyzed using two-way analysis of variance (ANOVA) with repeated measures for time combined with post-hoc analysis using Tukey's multiple comparison tests. A P value of < 0.05 was taken to indicate a statistical difference. Values are expressed as means ± standard error of the mean.

Chapter 3

Results

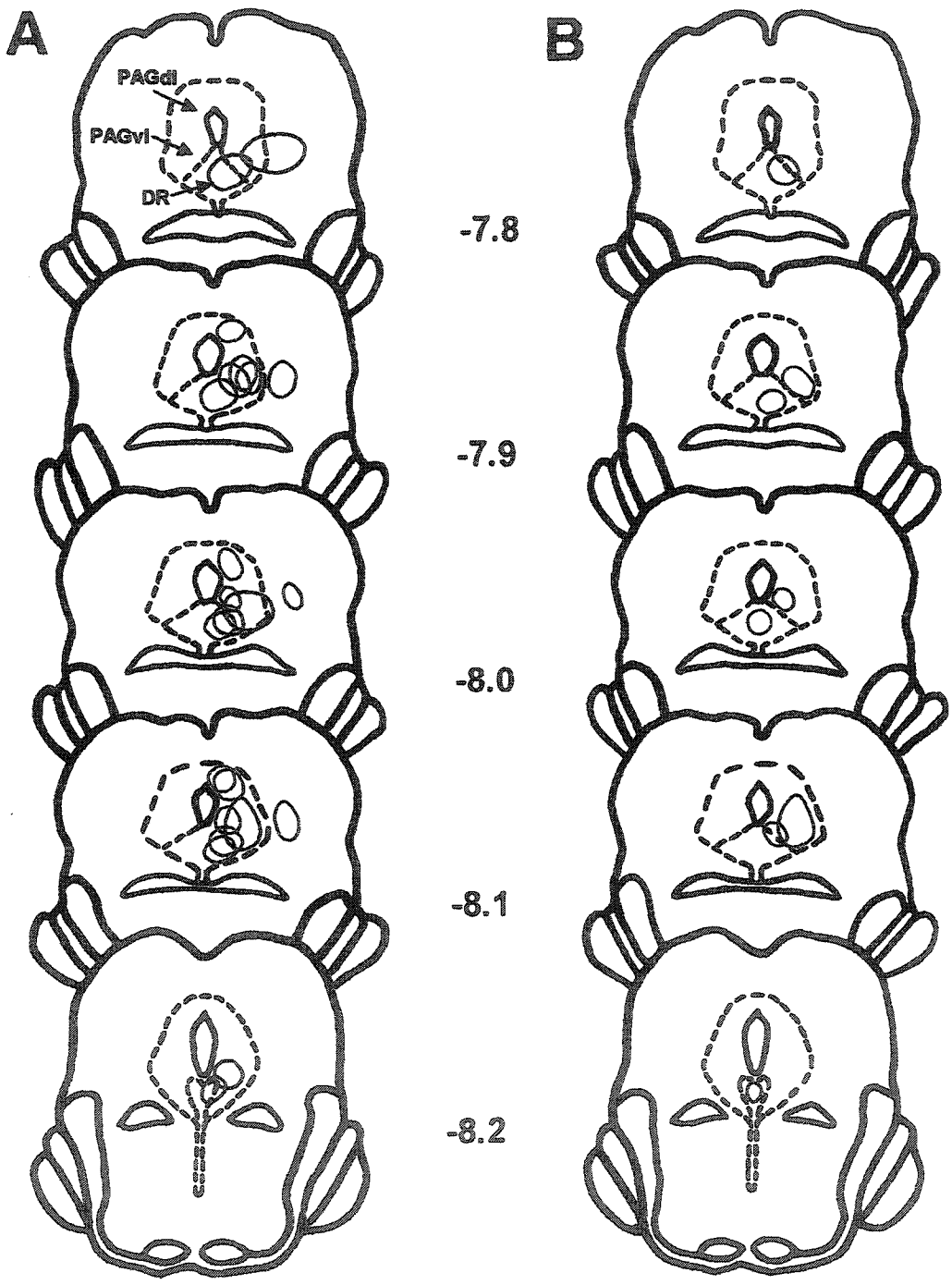
3.1 Retrograde Tracing of the VTA/SN Projection to the PAGvl/DR

3.1.1 Appearance of the CTb Injection Site

The center of the CTb injection site was darkly stained while the periphery appeared less intensive with a high density of granule and some fibers emanating from the injection site. The sites were generally round or ovoid and slightly variable in size as shown in Figure 4A. The diffusion of the CTb in brain tissue (the size of the injection site) is restricted by the density of cell bodies, the presence of blood vessels, and the location of fiber bundles within the injected regions. For example, the relatively cell dense region of the PAG limits the amount of diffusion of the CTb from injections in that area.

Tract-tracing experiments require the investigator to produce small but dense injections of tracers within a limited area of brain tissue. Iontophoretic

Fig. 4. Schematic diagram showing the location of cholera toxin B injections in the periaqueductal gray region and the dorsal raphe nucleus for the immunohistochemical tract-tracing experiments (A) or the double labeling immunofluorescence experiments (B). Numbers represent the anterioposterior levels of Paxinos and Watson. DR, dorsal raphe nucleus; PAGdl, dorsolateral periaqueductal gray; PAGvl, ventrolateral periaqueductal gray.


A
B
PAGdl
PAGvl
DR
-7.8
-7.9
-8.0
-8.1
-8.2

injections are well suited for this, but the ability to make successful injections of CTb by iontophoresis can be unreliable. Therefore technical problems of reliability were addressed by doing injections with micropipettes of different tip diameters. I found that the micropipette is easily blocked as it is lowered in the brain tissue when the tip diameter is greater than 30 µm, which results in a failure to inject CTb. A tip diameter of less than 15 µm always results in a small injection sites and fewer detectable retrogradely labeled cells. According to Luppi et al. (1990), CTb can be taken up and transported by damaged fibers passing in the areas of the micropipette tract. In other words, the smaller the pipette, the less likely it is that the CTb retrogradely labeled neurons result from CTb being taken up and transported by damaged fibers. Therefore, all of my CTb injections were done by using micropipettes with a relatively small tip of 15-25 µm.

I also tested a variety of concentrations of anti-CTb antibody (1:10,000 to 1:40,000) to maximize neuronal labeling and minimize the background staining. Lower concentration, for example 1:40,000, always gave darkly staining cells with clearly labeled cell bodies and dendrites. Higher concentration such as 1:10,000 gave dark cells with a high background staining. Therefore, most of the CTb immunohistochemical reactions were done in the concentration of 1:40,000 in both the single- and the double-labeling experiments. It should be pointed out that even with higher background staining (1:10,000), neurons were intensely stained black and could easily be distinguished from unlabeled neurons.

3.1.2 Single-labeling CTb Experiments

CTb injections were done iontophoretically in various portions of the caudal half of the PAG and parts of the DR embedded in the PAG (Fig. 4A). The injections were subdivided into different groups according to the extent of diffusion of the tracer. For description of the data, I will consider injections that were largely confined to the PAGvl (n = 8), DR (n = 4) and injections involving the wings of the DR and parts of the PAGvl (n = 3). Control injections of CTb were also made in the dorsolateral region of the PAG (n = 4) and laterally outside of the PAG in the nucleus cuneiformis and deep mesencephalic nucleus (DpMe, n = 4). In each group, the distribution of labeled cells was very similar for injections in specific regions of the PAG (for example, PAGvl compare to DR injections). Therefore, the data from one rat of each group (PAGvl, DR, PAGdl, outside PAG) is representative of each injection group.

Figure 5A, 6F and 11A show the distribution of neurons labeled in the midbrain following an injection of CTb that was largely restricted to the PAGvl. In this PAGvl injection, there was a little involvement of the lateral wing of the DR or the dorsolateral PAG, and no diffusion to the areas outside the PAG such as the nucleus cuneiformis or the DpMe. Retrogradely labeled neurons were found bilaterally in the rostrocaudal extent of the PAG as well as areas around the medial lemniscus. A large number of retrogradely labeled neurons were found in the SN below the medial lemniscus (Fig. 5, 6, 11D, 11E, 11F) in both the SNC

Fig.5. Coronal sections of the midbrain following an immunohistochemical reaction to show a cholera toxin B injection in the ventrolateral periaqueductal gray (A) and the neurons in the substantia nigra (B, C, D) and the deep mesencephalic nucleus (C) that were retrogradely labeled for cholera toxin B (black). A large number of labeled neurons were found in the compact and reticular part of the substantia nigra and the deep mesencephalic nucleus. DpMe, deep mesencephalic nucleus; DR, dorsal raphe nucleus; ML, medial lemniscus; PAGvl, ventrolateral periaqueductal gray; SNR, reticular part of the substantia nigra.

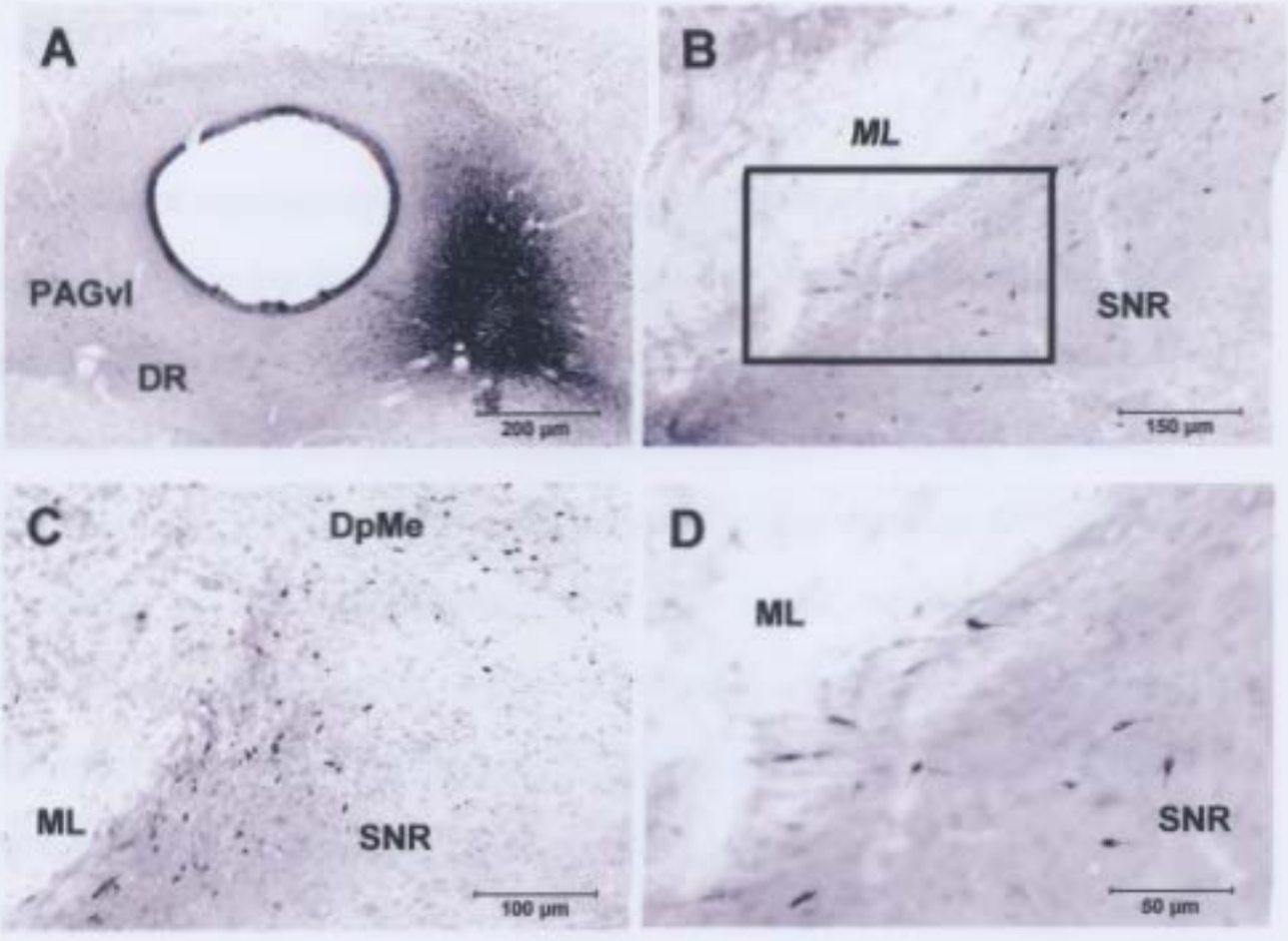
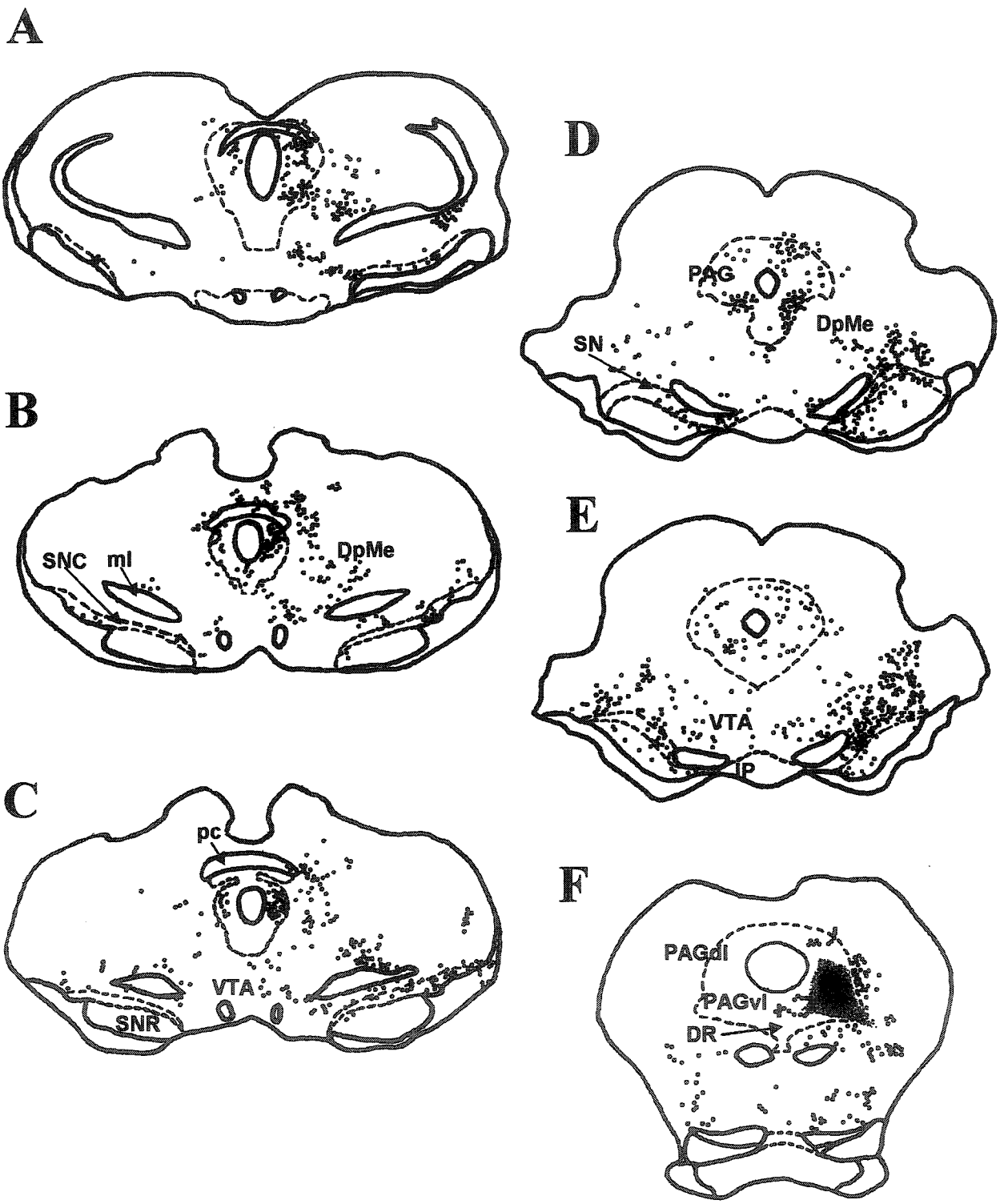

A
PAGvl
DR
200 µm
B
ML
SNR
150 µm
C
DpMe
ML
SNR
100 µm
D
ML
SNR
50 µm

Fig.6. A series of representative projection drawings of coronal sections through the midbrain showing the distribution of retrogradely labeled neurons following an injection of cholera toxin B into the ventrolateral periaqueductal gray (PAGvl). A large number of cells in the substantia nigra and a few in the ventral tegmental area project to the PAGvl. DpMe, deep mesencephalic nucleus; DR, dorsal raphe nucleus; IP, interpeduncular nucles; ml, medial lemniscus; PAGvl, ventrolateral periaqueductal gray; PAGdl, dorsolateral periaqueductal gray; pc, posterior commissure; SNC, compact part of the substantia nigra; SNR, reticular part of the substantia nigra; VTA, ventral tegmental area.


A
B
SNC
ml
DpMe
C
pc
VTA
SNR
D
PAG
DpMe
SN
E
VTA
IP
F
PAGdl
PAGvl
DR

and SNR. Even though a number of CTb-labeled neurons were found scattered in various regions in the rostrocaudal extent of the VTA, most of them were located in the caudal aspect of the VTA (Fig. 6, 11B, 11E). A large number of neurons were also retrogradely labeled in the SNL and the DpMe located dorsal to the SNL (Fig. 5C, 6, 11C).

Figure 7 shows a representative case of retrograde labeling following an injection of CTb confined to the DR. There are also some injections that are in the midline region of the DR (see Fig. 4 for example). As before, the projection is bilateral in origin with larger numbers of cells found in the VTA (Fig 7B to 7E) than what was observed following injections of CTb in the PAGvl (Fig. 6). This midline to lateral arrangement in the VTA/SN was consistently seen in the injections that involved either the DR or PAGvl. Similar to the injection of the PAGvl, CTb-labeled neurons are more numerous in the caudal part of the VTA than in the rostral part. Neurons were also labeled in regions around the medial lemniscus including in the region of the SNC and the dorsomedial aspect of the SNR.

Injections of CTb into the dorsolateral region of the PAG resulted in the retrograde labeling of a few neurons in the VTA or SN (Fig. 8). As with injections in the PAGvl and DR, CTb injections in the dorsolateral PAG resulted in a large number of CTb-labeled neurons in the lateral region of the DpMe. A sparse distribution of CTb-labeled neurons was found in the SNL, and only a few isolated CTb-labeled neurons were found in the VTA or SNC/SNR (Fig. 8).

Fig.7. Another series of representative projection drawings of coronal sections through the midbrain showing the distribution of retrogradely labeled neurons following an injection of cholera toxin B into the dorsal raphe nucleus. The dorsal raphe nucleus receives a dense projection from neurons in the ventral tegmental area and the compact part of the substantia nigra region.

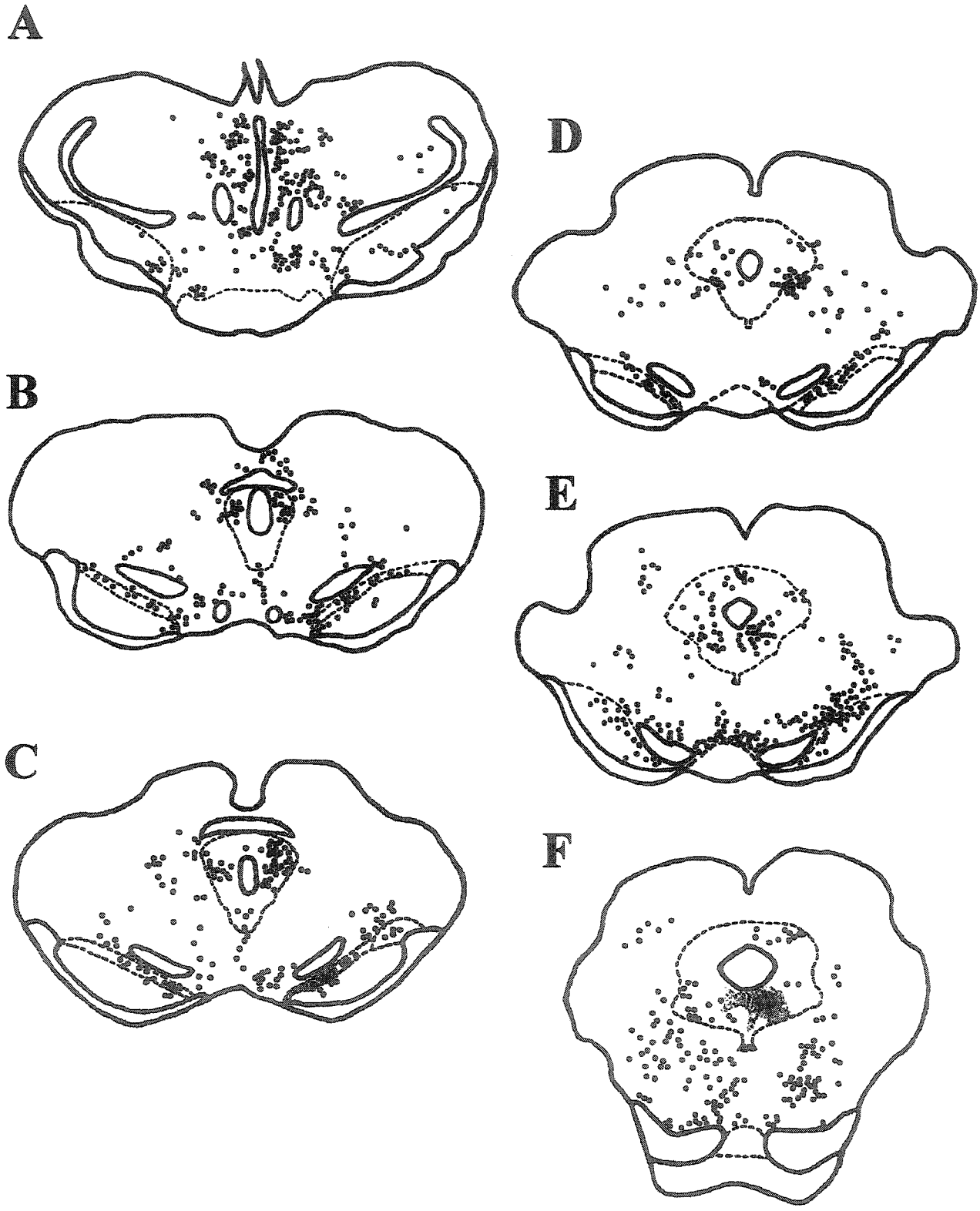
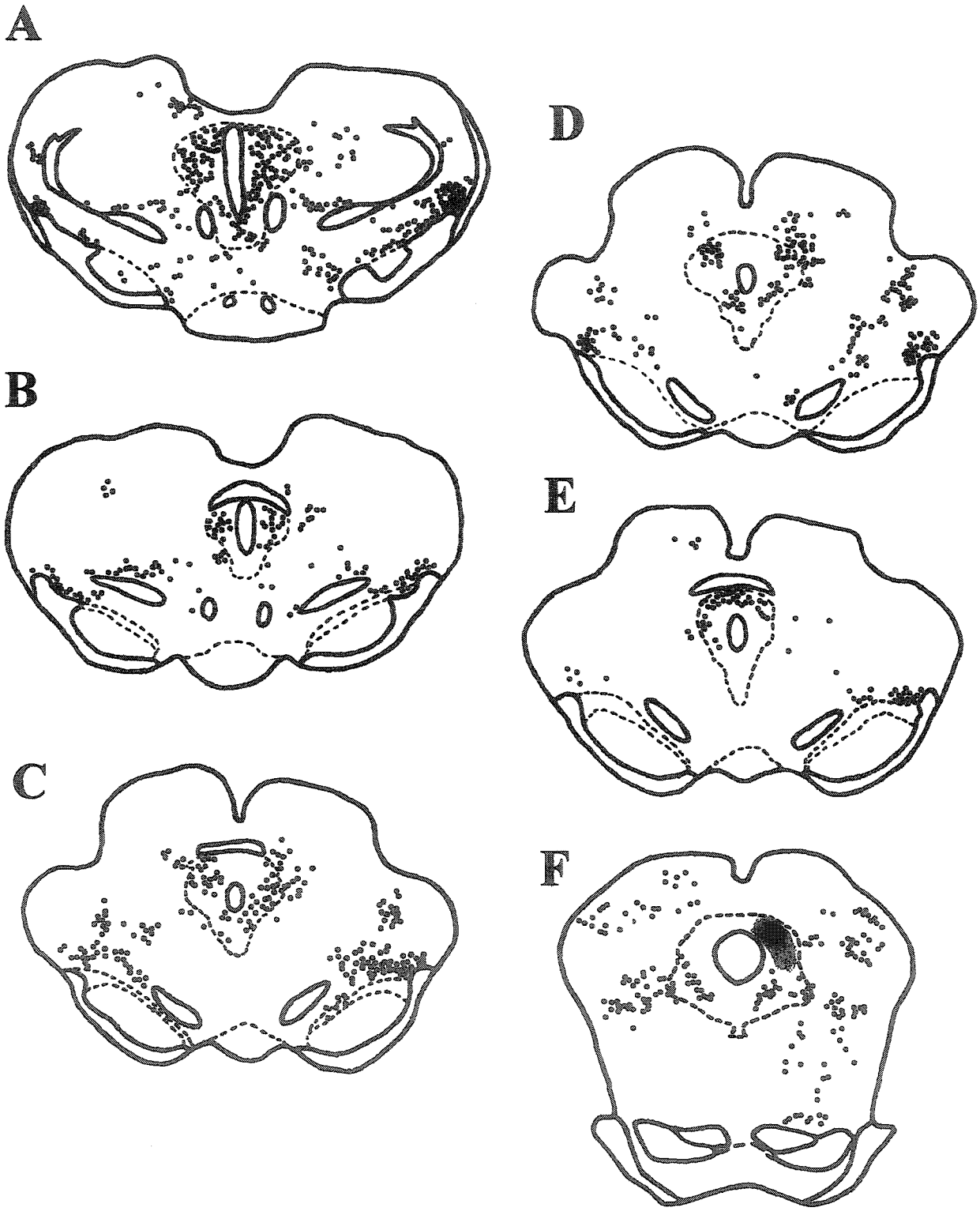

A
B
C
D
E
F

Fig. 8. Drawings of midbrain sections showing the distribution of retrogradely labeled neurons following an injection of cholera toxin B (CTb) into the dorsolateral periaqueductal gray (PAGdl). Cells in the PAG area project to the injection site, and a large number of CTb-labeled cells in the deep mesencephalic nucleus and a few cells in the lateral part of the substantia nigra project to the PAGdl.

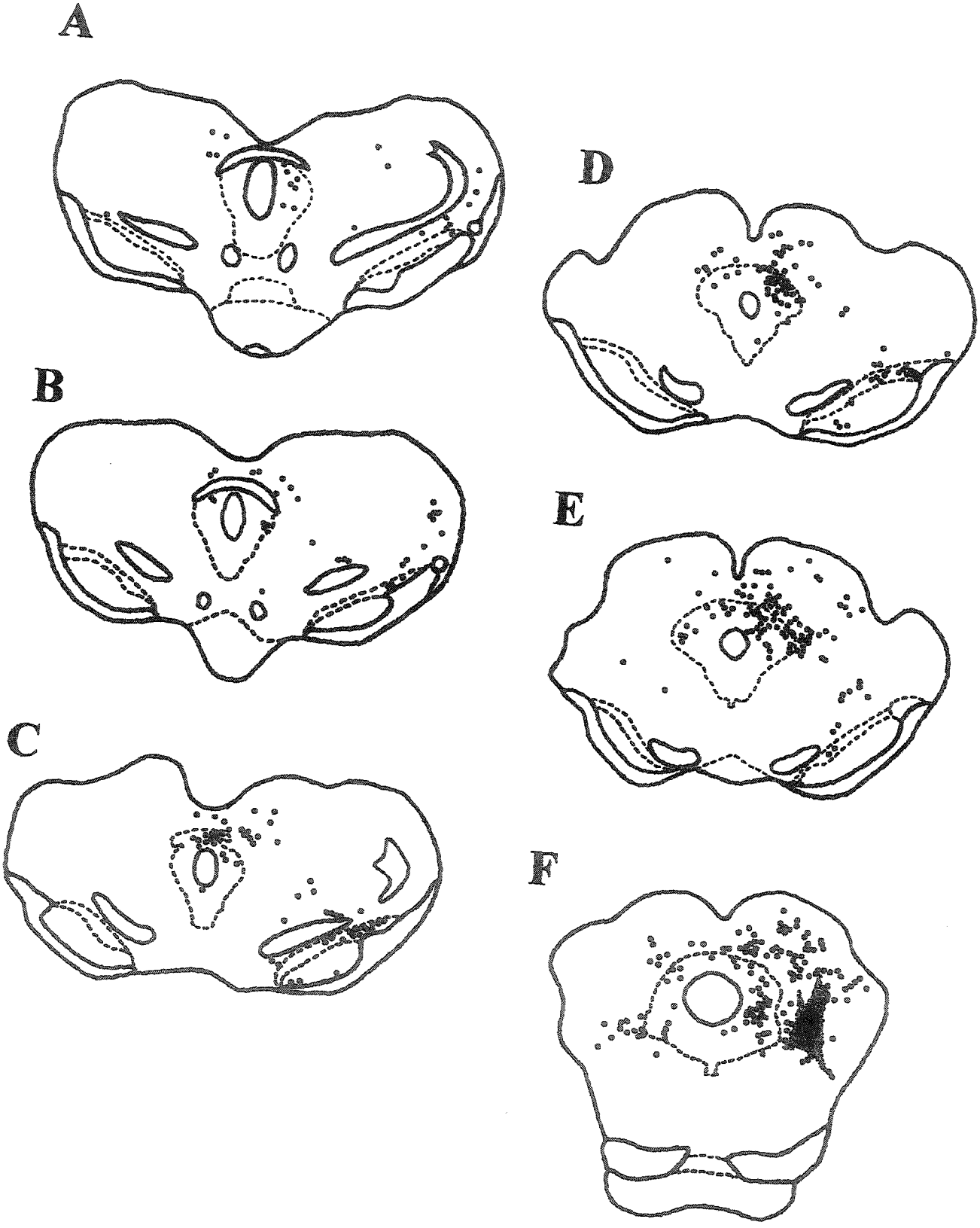

A
B
C
D
E
F

Control injections of CTb outside the PAG in the nucleus cuneiformis and DpMe resulted in retrograde labeling in mostly the SNL ipsilaterally and DpMe region immediately adjacent to the SNL (Fig. 9). CTb-labeled neurons were not seen in the rostrocaudal VTA.

3.2 Double-labeling Experiments to Determine Neurotransmitter

Double-labeling experiments were done with both immunohistochemistry and immunofluorescence to determine the putative neurotransmitter contained in the projection (dopamine or GABA). Double-labeling experiments using immunohistochemical protocols involve the use of DAB as a peroxidase substrate with and without nickel intensification (viewed with a light microscope). Double labeling with the immunofluorescent technique involved using Cy2 and Cy3 conjugated secondary antibodies to show labeling of different substances (CTb and neurotransmitter, viewed under a fluorescent microscope).

Fig. 9. Drawings of midbrain sections showing the distribution of retrogradely labeled neurons following an injection of cholera toxin B into the deep mesencephalic nucleus and nucleus cuneiformis. The ventral tegmental area does not project to nucleus cuneiformis area, and only a few isolated cells in the ipsilateral lateral part of the substantia nigra (SNL) and the deep mesencephalic nucleus region immediately adjacent to the SNL project to this area.


A
B
C
D
E
F

3.2.1 Double-labeling Experiments for Immunohistochemistry

Successful double-labeling protocols with DAB immunohistochemistry have been used by previous investigators. For double-labeling using CTb, Luppi et al. (1990) reported in a methodological paper that the histochemical identity of the retrogradely labeled cells can be identified based on the differential staining of CTb and neurotransmitters in the cell bodies. For example, CTb+ neurons stained by DAB-nickel intensificaiton appear as black punctate granules because the CTb is concentrated in vesicles in the cell bodies. GAD+ neurons appear evenly brown when reacted with DAB without nickel intensification because GABA is homogenously distributed in the cell body. Therefore, the double-labeled cells can be recognized by the presence of black punctate granules (CTb) over a brown diffusely stained cytoplasm (GAD).

In my experiments, GAD immunohistochemistry resulted in a relatively high background staining. It was hard to distinguish which neurons were black CTb+ neurons and which were brown GAD+ neurons. In an attempt to decrease the high background staining, brain sections were pretreated with 1% solution of sodium borohydride in 0.1M PBS for 10 min or 3% solution of hydrogen peroxide in 0.1M PBS for 10 min. However, these approaches did not help in reducing the high GAD background staining. This suggested that the high background staining might be due to other factors.

Esclapez et al. (1994) reported that the relatively high background staining in the VTA/SN was due to the high level of GAD in fibers and axon terminals in the ventral midbrain region, especially in the SNR region where GABA fibers were found in greatest numbers. I tried to use lower concentrations (from 1:1000 to 1:10,000) of the primary GAD antibody in several of my double-labeling experiments. However, problems of clearly identifying double-labeled neurons were still present. The approach of demonstrating double-labeled CTb/GAD neurons using light microscope and DAB as a chromogen was considered inappropriate for the midbrain. Others have reported similar problems (González-Hernández, and Rodríguez, 2000; Rodríguez, *et al.*, 2001).

Glutamic acid decarboxylase (GAD) is the enzyme responsible for the synthesis of GABA in nervous system. There are two isoforms that have been identified in the central nervous system, GAD65 and GAD67, each of which is encoded by a different gene. There are many differences between these two GAD proteins including amino acid sequence, molecular size, their interaction with the co-factor pyridoxal 5'-phosphate, and their regulatory control (Erlander and Tobin, 1991). GAD65 is localized in membrane-associated neuronal compartments such as synaptic vesicles (Mclaughlin *et al.*, 1975; Reetz *et al.*, 1991) and β-cell synaptic-like microvesicles (Reetz *et al.*, 1991), whereas GAD67 is a soluble cytosolic protein (Christgau *et al.*, 1992; Solimena *et al.*, 1993). In many brain regions, the two isoforms GAD65 and GAD67 are not evenly distributed: GAD65 is predominantly present in the axon terminals while GAD67

is more highly concentrated in the cell bodies (Esclapez *et al.*, 1994; Dirkx *et al.*, 1995). González-Hernández and Rodríguez (2000) have reported that no GAD65 labeled cells were found in the SN, which suggested that this GAD isoform was localized in axonal terminals. Using detergent Triton X-100 in GAD immunohistochemistry resulted in a decreased staining of cell bodies and an increased staining of axon terminals (Esclapez *et al.*, 1994). This observation suggests that, because Triton X-100 increases the membrane permeability, more GAD antibodies penetrate and bind to the membrane-associated GAD65 in the synaptic vesicles or plasma membranes than those that bind to the soluble GAD67 in cytoplasm. I observed during my experiments that a low concentration of Triton X-100 is necessary for CTb antibodies to penetrate adequately. Therefore, a lower concentration of 0.01% Triton X-100 was used for CTb/GAD double-labeling immunofluorescent experiments.

Problems with identifying double-labeled CTb/TH neurons also occurred when double-labeling experiments were attempted due to the very high density of TH immunoreactive neurons and fibers in the VTA and SN. TH is found in very high concentration in dopamine neurons and labeling with TH immunohisto-chemistry produces intensely stained neurons which obscured the CTb labeling. Results of double-labeling experiments with DAB immunohisto-chemistry are not presented here because of the numerous methodological problems associated with the technique. This is largely due to the chemical nature of the VTA/SN and

not the technique itself, which has been successfully used in other regions of the brain.

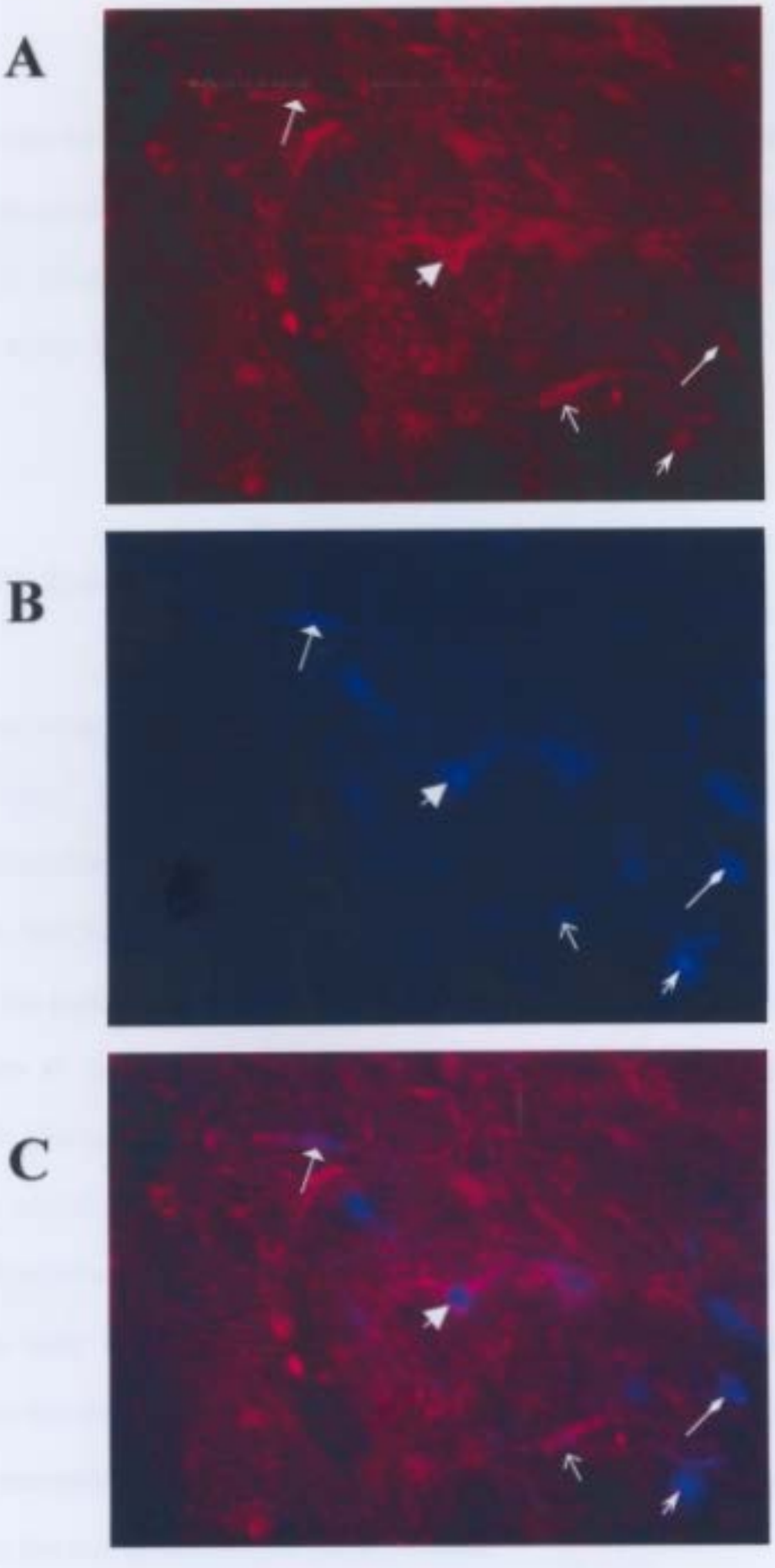
3.2.2 Double-labeling Experiments for Immunofluorescence

3.2.2.1 FluroGold Experiments

In double-labeling experiments using FluroGold (FG), FG-labeled neurons appeared whitish blue when examined under the UV filter (see Fig. 10B), and Cy_3 conjugated donkey anti-rabbit immunoreacted GAD+ neurons appeared red (Fig. 10A) under green filter. As described above, the FG-labeled cells were whitish blue while the GAD+ neurons could not be observed under UV filter. But under the U-MNG2 filter, GAD+ and FG-labeled neurons both appeared red, because FG fluoresces under a wide spectrum and can be seen with the U-MNG2 filter cube. Therefore, it was difficult to tell if a cell is a GAD+ neuron when the same neuron contains a large amount of FG.

In addition, cells that are considered double-labeled should not only have the same location in the sections, but also the same size and shape. Looking for double-labeled neurons required switching filters several times to make sure that the neuron was double-labeled. However, FG fades very quickly when examined with fluorescent microscope. Therefore, it was difficult to examine the tissue for double-labeled neurons without having the fluorescent probes fade because of

Fig. 10. Series of photomicrographs showing glutamic acid decarboxylase (GAD)+ neurons (A) and FluroGold-labeled neurons (B) in the ventral tegmental area. Panel C represents a merged image from panels A and B, and shows neurons that were double labeled for FluroGold and GAD (arrows).


A
B
C
50μm

the time it took to make a positive identification. This approach would likely lead to both false positive and false negative identification of double-labeled neurons. Because of these limitations, the decision was made not to use FG as a retrograde tracer for the immunofluorescent experiments.

3.2.2.2 CTb Experiments

Immunohistochemical experiments were done with a variety of GAD and TH antibodies. Polyclonal antibodies against GAD (catalog number AB108, AB1151, Chemicon International, Temecula, CA; catalog number 228485, Alpha Diagnostic, San Antonio, TX, USA) resulted in labeling of neurons in the VTA/SN. However, the background staining with these GAD antibodies interfered with the visualization of cell body staining. Polyclonal antibody immunohistochemistry against TH (Serotec, Raleigh, NC) resulted in inconsistent outcomes and weak cell body staining. Use of monoclonal antibodies against GAD and TH (Chemicon International, Temecula, CA and Sigma, respectively) resulted in very strong cell body staining with low background. After several experiments to standardize the protocols the decision was made to use monoclonal antibodies at lower concentrations (GAD, 1:500 and TH, 1:40,000), which gave the best labeling for the immunofluorescent experiments.

Fig. 11. Photomicrograph of cholera toxin B (CTb) injections in the ventrolateral periaqueductal gray (A) and retrogradely CTb labeled neurons in relation to tyrosine hydroxylase (TH) labeled neurons. These plates were produced by merging two images taken under different filters to show the green Cy2-labeled CTb and red Cy3-labeled TH. A few CTb-labeled neurons were found in the ventral tegmental area (B, E) whereas, a large number of CTb-labeled neurons were found in the ventral region of the compact part of the substantia nigra (D, E, F) where TH-labeled neurons are located and in the reticular part of the substantia nigra (B, C, D) immediately ventral to the TH-labeled neurons. There are no neurons that are double-labeled for CTb and TH. DpMe, deep mesencephalic nucleus; DR, dorsal raphe nucleus; ML, medial lemniscus; PAGvl, ventrolateral periaqueductal gray; PAGdl, dorsolateral periaqueductal gray; SNC, compact part of the substantia nigra; SNR, reticular part of the substantia nigra; VTA, ventral tegmental area.

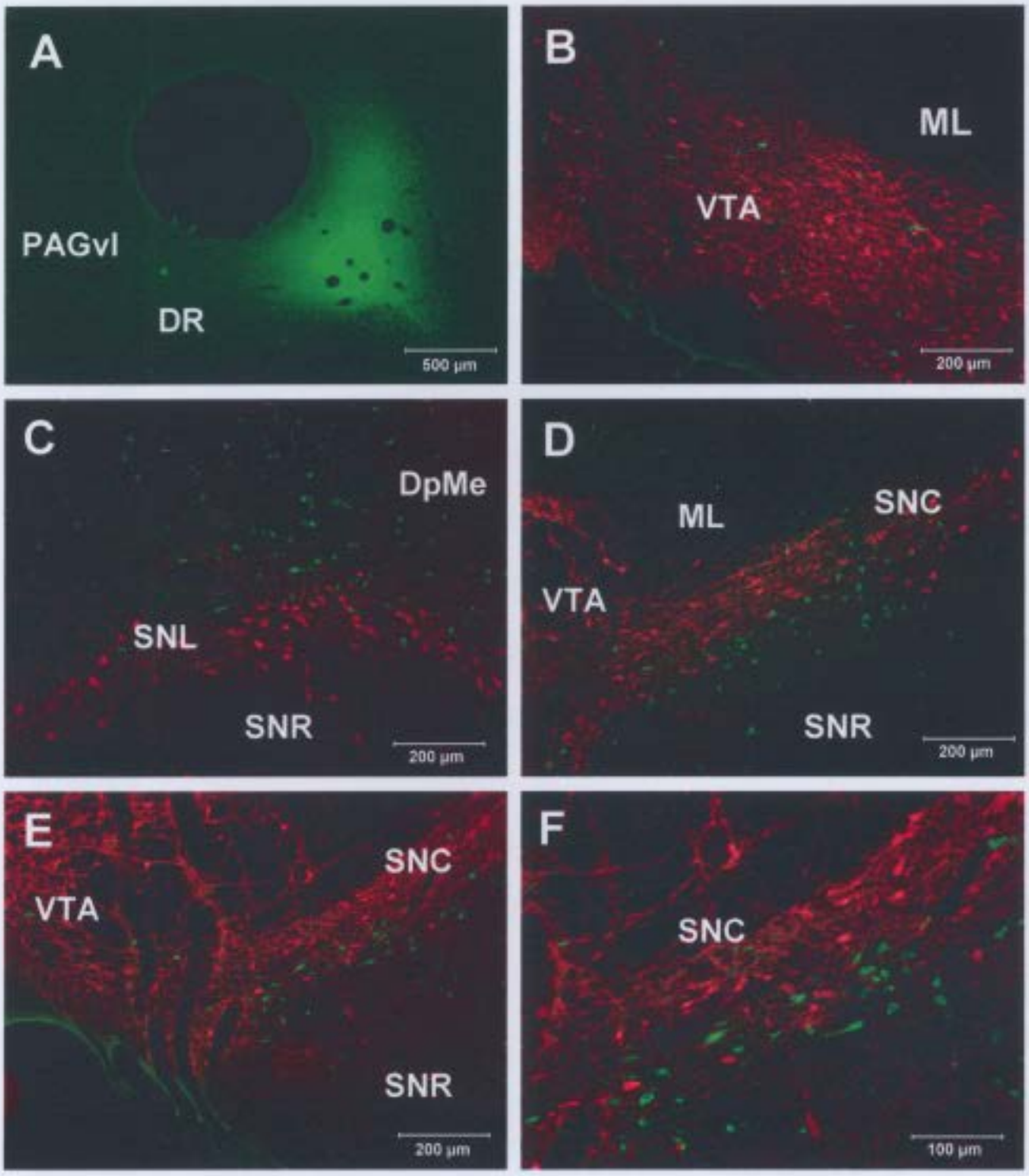
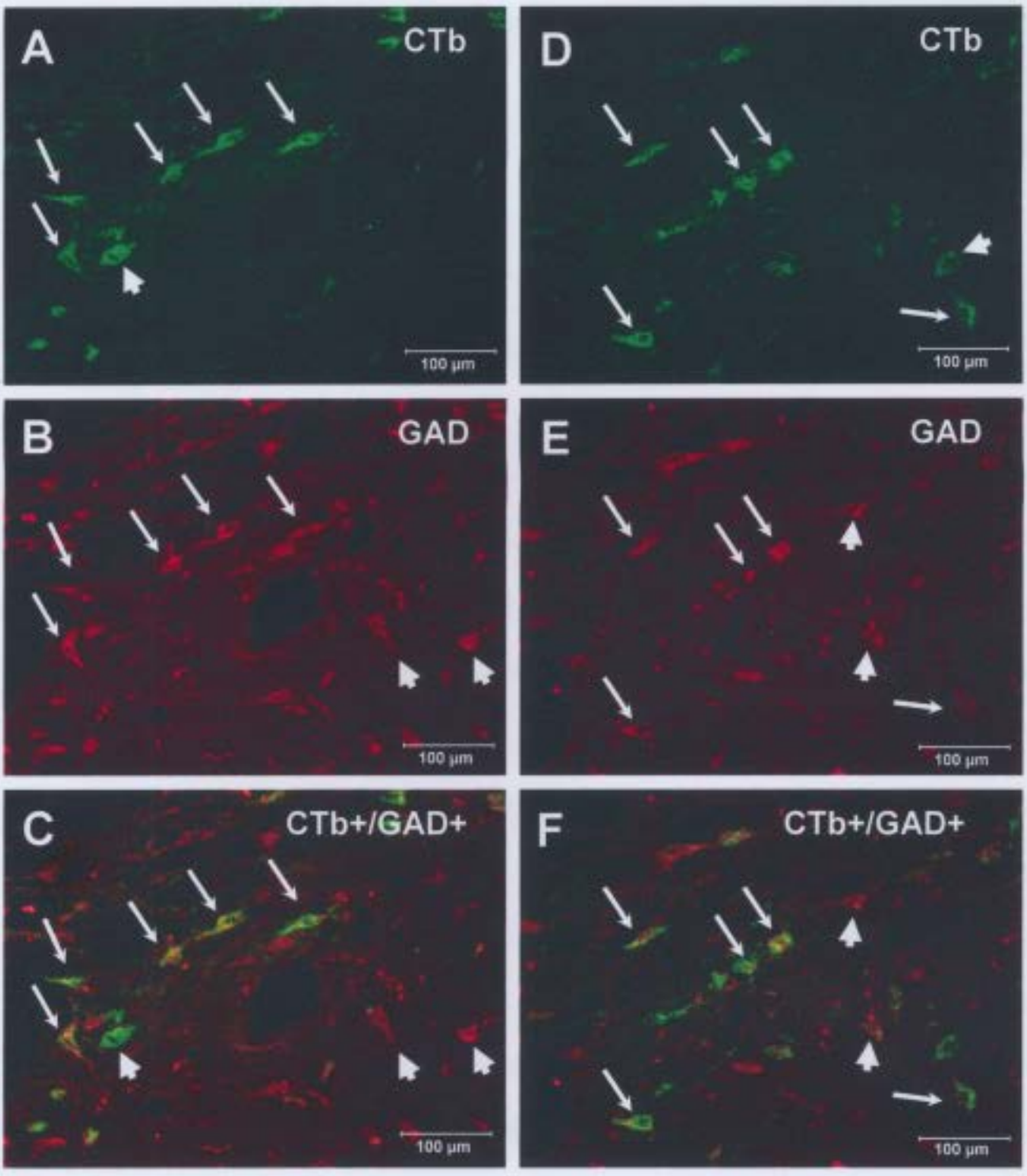

A
PAGvl
DR
500 µm
B
ML
VTA
200 µm
C
DpMe
SNL
SNR
200 µm
D
ML
SNC
VTA
SNR
200 µm
E
SNC
VTA
SNR
200 µm
F
SNC
100 µm

Figure 4B illustrates the location of the CTb injections used for these double-labeling immunofluorescent experiments (n = 8). The injections were restricted to either the PAGvl or the DR, or involved a combination of these regions. Figure 11 shows the distribution of CTb-labeled neurons in the VTA (Fig. 11B, 11E) and SN (Fig. 11D, 11E, 11F) following injections of CTb in the PAGvl/DR region (Fig. 11A) relative to TH+ neurons. CTb-labeled neurons that project to the PAGvl/DR were found interspersed with TH+ neurons in the VTA and SN. A considerable overlap in the location of the CTb-labeled and TH+ neurons was found in the transition zone between the SNC and SNR (Fig. 7F). However, when sections were examined at higher magnification, CTb-labeled neurons that were double labeled for TH were not observed (in 4 rats with CTb injections in the PAGvl/DR). Counts for CTb and TH double-labeled cells are not reported here because there was no evidence of double labeling for these markers.

Tissue sections immunoreacted for both CTb and GAD neurons showed a proportion of neurons double labeled for both CTb and GAD (see Fig. 12 for example). Double-labeled neurons (those that were retrogradely labeled by CTb and labeled for GAD) were counted in four representative levels of the VTA/SN in 4 rats, which had injections limited to the PAGvl/DR. The numbers of CTb+/GAD+ neurons were 47/15 (32%) for the VTA; 198/75 (38%) for the SNR; 78/7 (9%) for the SNC; 167/19 (11%) for the DpMe. It is possible that this represents an underestimation of the total number of CTb+/GAD+ neurons

Fig.12. Photomicrographs of cholera toxin B (CTb) and glutamic acid decarboxylase (GAD) labeled neurons in the reticular portion of the substantia nigra following an injection of CTb into the ventrolateral periaqueductal gray (PAGvl, Fig. 11A). Plates A and D show CTb-labeled neurons viewed with a filter to show Cy2-labeled CTb neurons. Plates B and E show the same region as A and D but viewed with a filter to show Cy3-labeled GAD+ neurons. Plates C and F show merged images (A and B) and (D and E), respectively. Small arrows indicate double-labeled neurons whereas the large arrow heads indicate single CTb or GAD labeling.


A
CTb
100 µm
D
CTb
100 µm
B
GAD
100 µm
E
GAD
100 µm
C
CTb+/GAD+
100 µm
F
CTb+/GAD+
100 µm

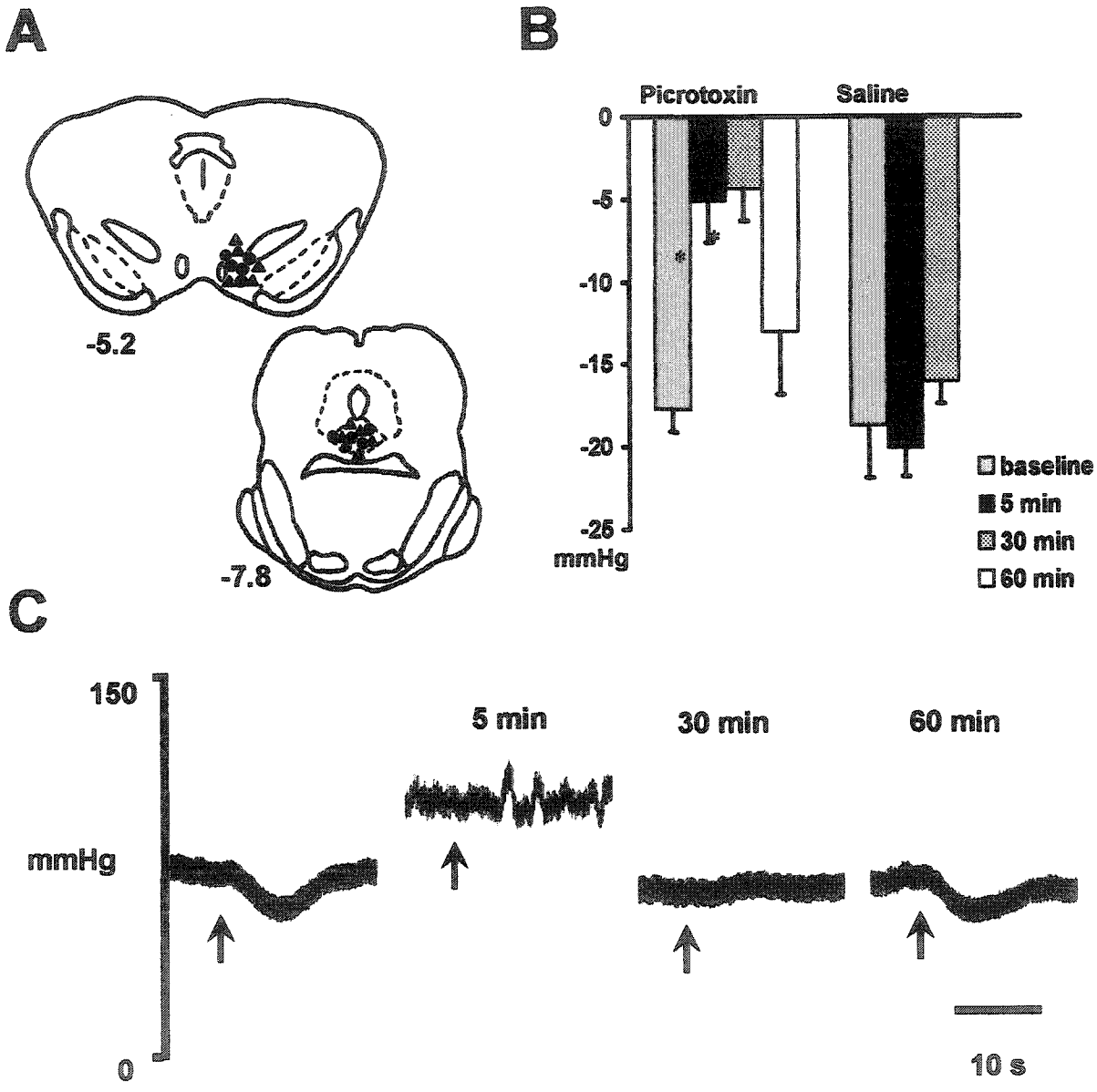
making up the projection because a number of CTb+ neurons were weakly stained for GAD and were not considered as double-labeled neurons (see Fig. 12 for examples).

3.3 Physiological Experiments

Picrotoxin is a pharmaceutical agent that selectively blocks the chloride channels by interacting with the GABA-receptor-ionophore complex. Injections of picrotoxin in the PAGvl/DR produced no immediate cardiovascular responses. However, it did result in a trend towards an increase in MAP that did not reach significance level (pre-picrotoxin 82.8 ± 4.2 mmHg; 5 min post-picrotoxin 99.7 ± 6.5 mmHg; 30 min post-picrotoxin 94.2 ± 7.1 mmHg; n = 5) which was more apparent in some experiments (Fig. 13). In some cases, brief increases in respiratory activity following injections of picrotoxin occurred with the increases in MAP. The ANOVA indicated that injections of picrotoxin attenuated the depressor responses elicited by activation of the VTA/SN with glutamate ($F_{3,19}$ = 3.238, $P < 0.008$; Fig. 9). The baseline response to VTA/SN stimulation was attenuated by 69% at 5 min ($P < 0.05$, n = 5, Fig. 13B, 13C) and by 74% at 30 min ($P < 0.05$, n = 5, Fig. 13B, 13C) post-picrotoxin administration. Restimulation of the same site in the VTA/SN at 60 min post-picrotoxin resulted in depressor

response of similar magnitude as the baseline response (Fig. 13B, 13C). Stimulation of the VTA/SN produced small and inconsistent changes in HR responses that were unaffected by picrotoxin injections in the PAGvl/DR (data not shown). The sites of the picrotoxin injections were verified on histological sections to be located in the caudal portion of the PAGvl/DR region (Fig. 13A). Control injections of saline in the PAGvl/DR had no effect on the cardiovascular depressor responses elicited from repeated stimulation of the same site in the VTA/SN (n = 5; Fig. 13B).

Fig. 13. Effects of picrotoxin on cardiovascular depressor responses from stimulation of the ventral tegmental area/substantia nigra (VTA/SN). A: Drawings of the midbrain showing the site of injections of glutamate in the VTA/SN (A) or injections of picrotoxin/saline in ventrolateral periaqueductal gray/dorsal raphe nucleus (PAGvl/DR) (B) for the picrotoxin (triangle) and control saline (circle) experiments. B: Histogram shows the effect at 5, 30 and 60 min compared with baseline responses after the administration of picrotoxin (n = 5) or saline (n = 5) in the PAG/DR on the depressor response elicited from stimulation of the VTA/SN. * $P<0.05$. C: A representative experiment showing the effect of microinjections of picrotoxin in the PAG/DR on the magnitude of the arterial pressure response elicited from stimulation of the VTA/SN. The tracings show the magnitude of the response to stimulation of the same site in the VTA/SN at 5, 30, and 60 min post-injection of picrotoxin in the PAG/DR. Note that the depressor response at 60 min post-picrotoxin was of similar magnitude as the pre-picrotoxin depressor response elicited by stimulation of the VTA/SN.


A
0
-5.2
-7.8
B
Picrotoxin
Saline
0
-5
-10
-15
-20
-25
mmHg
baseline
5 min
30 min
60 min
C
150
mmHg
0
5 min
30 min
60 min
10 s

Chapter 4

Discussion

Four major observations can be made from results of the present study:

1. the PAGvl and DR receive a substantial input from neurons in the VTA, SN, and DpMe.
2. this projection does not appear to use dopamine as a transmitter substance (TH negative).
3. a portion of VTA and SN neurons projecting to the PAGvl/DR likely use GABA as a transmitter substance (GAD positive).
4. the cardiovascular depressor responses elicited by activation of neurons in the VTA/SN appear to be mediated by GABA release in the PAGvl/DR because picrotoxin injections in the PAGvl/DR blocked the responses.

4.1 Anatomical Experiments

4.1.1. Projection from the VTA/SN to the PAGvl/DR

The neuronal connections between the VTA/SN and the PAGvl/DR have been reported in previous tract tracing studies, but the details of this projection have not been the subject of a detailed investigation (Sakai *et al.*, 1977; Beckstead *et al.*, 1979; Simon *et al.*, 1979; Beitz *et al.*, 1982; Marchand *et al.*, 1983; Kalén *et al.*, 1988; Peyron *et al.*, 1995; Gervasoni *et al.*, 2000; Kirouac & Pittman, 2000). Studies looking at afferent inputs to the PAG using older and likely less sensitive tract tracing methods reported a relatively weak projection from the VTA/SN to the PAG region (Sakai *et al.*, 1977; Beckstead *et al.*, 1979; Simon *et al.*, 1979; Beitz *et al.*, 1982; Marchand *et al.*, 1983). More recent studies, which have used more sensitive tracing substances such as CTb or fast blue, reported a moderately dense projection between the VTA/SN and the DR (Kalén *et al.*, 1988; Gervasoni *et al.*, 2000). In a recent investigation using an anterograde tracer to describe descending brainstem projection from the VTA/SN, Kirouac and Pittman (2000) demonstrated the presence of a dense bilateral projection from the VTA/SN to the PAGvl and the wings of the DR. The present investigation was done to describe the location of neurons in the VTA/SN that innervate to the PAGvl/DR and to identify the transmitter substance used by these neurons.

In the present investigation, we found that the projection from the VTA/SN to the PAGvl/DR showed a weak topography with the VTA preferentially innervating the wings of the DR while the SN preferentially innervating the PAGvl. However, there was considerable overlap in this arrangement, with neurons in the VTA and SN innervating both the PAGvl and the DR. A large number of neurons in the DpMe were also consistently found to send projections to both the PAGvl and the DR. Recent studies looking at afferent projections to the DR reported that neurons located in both the VTA and the SN innervated the DR (Kalén *et al.*, 1988; Gervasoni *et al.*, 2000). In contrast to a substantial innervation of the PAGvl/DR, neurons in the VTA and SN did not appear to innervate the dorsolateral regions of the PAG.

A finding worth noting in the present study was the large number of neurons in the DpMe innervating both the PAGvl and DR. Labeled fibers were reported in the PAG following injections of tritiated amino acids in the DpMe (Veazey and Severin, 1980a, b). However, identification of fiber terminals cannot be made using tritiated amino acids as an anterograde tracer. Therefore, the present study extends the work of Veazey and Severin (1980a, b) by demonstrating that a large number of neurons of the DpMe provide fiber terminals to the PAGvl/DR.

The DpMe, also termed the midbrain reticular formation, central tegmental field, nucleus mesencephalicus profundus, or cuneiformis/subcuneiformis complex (Huber *et al.*, 1943; Olszewski and Baxter, 1954; Taber, 1962; Valverde,

1962), is a large midbrain region that composed of small, medium, and large-sized neurons (Huber *et al.*, 1943). The DpMe is located ventrally to the superior colliculus, dorsally to the SN, laterally to the red nucleus and the PAG, and medially to the medial geniculate nucleus (Veazey and Severin, 1980a, b, 1982; Hay-Schmidt and Mikkelsen, 1992; Yasui *et al.*, 1994). The DpMe has been divided into a lateral division that sends ascending fibers to the basal ganglia and a medial division that sends descending projections to different nuclei of the caudal brainstem (Veazey and Severin, 1980a, b, 1982; Hay-Schmidt and Mikkelsen, 1992; Yasui *et al.*, 1994).

The DpMe has been considered to be involved in nociception, cardiovascular regulation (Nestianu and Mihailescu, 1985; Gonzalez-Lima, 1988; Wang *et al.*, 1992), and basal ganglia functions (Rodriguez *et al.*, 2001). Both the SN and the DpMe contain GABA neurons (Mugnaini and Oertel, 1985; Appell and Behan, 1990; Esclapez *et al.*, 1993). It is known that the SNR contains GABA neurons and is considered an important output nucleus of the basal ganglia (Albin *et al.*, 1989; Alexander and Crutcher, 1990; Obeso *et al.*, 1997). Recently, some studies have shown that the DpMe receives inputs from striatum as well (Rodriguez *et al.*, 2001). Several studies also reported that the DpMe projects to the ventral thalamus and different midbrain and brainstem nuclei such as the superior colliclulus, pedunculopontine tegmental nucleus, medullar reticular nucleus, and central gray (Veazey and Severin, 1980a, b, 1982). These

data suggest that the DpMe may play a role in the basal ganglia similar to that of the SNR.

A small proportion of neurons (11%) in the DpMe projecting to the PAGvl/DR were found to be GAD+. This suggests that the projection from the DpMe to the PAGvl/DR uses more than one neurotransmitter because GABA only forms a portion of the projection. It should also be pointed out that the anatomy and function of the DpMe have not been the subject of much investigation except for recent studies (Rodríguez *et al.*, 2001; González-Hernández *et al.*, 2002). This may be an area of interest for future anatomical and physiological studies.

4.1.2. Putative neurotransmitters

Axons and terminals immunoreactive for dopamine have been described in the PAGvl/DR (Kitahama *et al.*, 2000). The location of dopamine neurons innervating the DR is likely to be regions of the hypothalamus and not the VTA or the SN (Kalén *et al.*, 1988; Peyron *et al.*, 1995). However, the source of dopamine neurons projecting to the PAGvl may not be the same as the source of projections to the DR. Based on our tract-tracing experiments that showed labeled neurons in the VTA and SNC (see Fig. 2 for example) following injections of CTb into the PAGvl, we examined the possibility that dopamine neurons in the

VTA or SN project to the PAGvl. Despite the apparent overlap of CTb neurons projecting to the PAGvl/DR and neurons stained with TH (Fig. 7), we did not find CTb neurons that were also labeled with TH. These results are consistent with previous studies reporting that the sources of dopamine fibers innervating the DR are the A11 and A13 dopamine neurons located in the dorsal hypothalamus (Kalén *et al.*, 1988; Peyron *et al.*, 1995).

Neurons in the VTA/SN have been shown, using immunohistochemical techniques, to contain the neuropeptides neurotensin and cholecystokinin (Seroogy *et al.*, 1988). However, these neuropeptides are not found in isolation in subpopulations of neurons in the VTA/SN but are colocalized with dopamine in the same neuron (Seroogy *et al.*, 1988). Since CTb+ cells were always found to be TH-, we would not expect CTb+ cells to contain either neurotensin or cholecystokinin.

The other well-known neuronal type found in the region of the VTA/SN is GABA (González-Hernández and Rodríguez, 2000). Therefore, the experiments addressed the possibility that GABA neurons in the VTA/SN innervate the PAGvl/DR. A proportion of neurons in the VTA (32%), SNC (9%), and SNR (38%) that projected to the PAGvl/DR contained GAD, an enzyme involved in the synthesis of GABA. This observation is consistent with a recent study showing that as many as 58% of the neurons in the VTA that projected to the DR were GAD positive (Gervasoni *et al.* 2000). The differences in the total number of GAD positive cells projecting to the PAGvl/DR noted by the two studies may be

due to the sensitivity of the different techniques used to show GAD positive neurons. For example, our study used immunofluorescence with a Cy_3 conjugated secondary antibody whereas Gervasoni et al. (2000) used the avidin-biotin complex method and the chromogen DAB. In the present study cells that were not clearly labeled for GAD in our counts were not included in order to eliminate the possibility of including false positives. Therefore, the counts in our study likely represent an underestimation of the proportion of GABA neurons in the VTA/SN that project to the PAGvl/DR.

A novel opioid peptide recently identified as having biological activities in the brain is nociceptin/orphanin FQ (Darland *et al.*, 1998; Taylor and Dickenson, 1998). This opioid peptide has been shown to be present in some neurons in the VTA/SN (Anton *et al.*, 1996) and could therefore be a potential candidate as a transmitter substance innervating the PAG. The PAG also contains a dense network of nociceptin/orphanin FQ fibers and terminals (Anton *et al.*, 1996; Norton *et al.*, 2002). In preliminary experiments done in our laboratory, we were unable to detect this opioid peptide using immunohistochemistry when done on midbrain tissue from colchicine treated rats (unpublished observations). Therefore, we cannot eliminate the possibility that a number of neurons in the VTA/SN innervating the PAG use nociceptin/orphanin FQ as a transmitter substance. It may be necessary to use *in situ* hybridization of mRNA to examine this question appropriately (Neal *et al.*, 1999; Norton *et al.*, 2002). It has also been reported that GAD and mRNA for nociception/orphanin FQ colocalize in the

same neurons (Norton *et al.*, 2002). It would be of interest to determine if GABA neurons in the VTA/SN that project to the PAGvl/DR also contain this opioid peptide. This is of particular importance since nociceptin/orphanin FQ in the PAG appears to play an important role in pain modulation (Darland *et al.*, 1998; Taylor and Dickenson, 1998).

4.2 Physiological Experiments and Anatomical Connections

Kirouac and Pittman (2000) provided evidence that the cardiovascular depressor responses produced by stimulation of the VTA/SN were mediated by a projection to the PAGvl/DR. This conclusion was based on experiments showing that reversible block of nerve impulse transmission and synaptic transmission in the PAGvl/DR with lidocaine and cobalt chloride, respectively, attenuated the depressor responses elicited by stimulation of the VTA/SN (Kirouac and Pittman, 2000). In the present study, blocking of chloride channels associated with GABA receptors with injections of picrotoxin into the PAGvl/DR region almost eliminated the cardiovascular depressor responses to stimulation of the VTA/SN. Therefore, the results of the physiological experiments support our hypothesis that a functional pathway exists between these two midbrain regions. In addition, the

results of our physiological experiments are consistent with our anatomical evidence showing that GAD+ neurons in the VTA/SN project to the PAGvl/DR. Moreover, the physiological and anatomical experiments support the view that activation of a VTA/SN GABAergic projection to the PAGvl/DR mediates the cardiovascular depressor responses produced by stimulation of the VTA/SN. However, it is also possible that the depressor responses from VTA/SN stimulation were eliminated because picrotoxin inhibited GABA interneurons in the PAGvl/DR, which are known regulators of PAGvl projection neurons (Reichling and Basbaum, 1990).

It is plausible that stimulation of the VTA/SN produces hypotensive responses by activating a projection from the PAGvl to the ventromedial and ventrolateral medulla (Lovick, 1993; Bandler and Shipley, 1994; Behbehani, 1995; Bandler *et al.*, 2000). The ventromedial and ventrolateral medulla project to preganglionic sympathetic neurons in the intermediolateral cell column and activation of these descending pathways can produce vasodilation in the circulation by inhibiting preganglionic sympathetic motor neurons (Dampney, 1994; Sun, 1995; see Fig. 3). Therefore, stimulation of the VTA/SN may in turn activate output neurons in the PAGvl that exert influence on cardiovascular regulatory centers in the medulla.

Numerous anatomical and physiological investigations have demonstrated that the neural circuitry in the PAGvl also mediates antinociception (see review by Behbehani, 1995). The rostral ventromedial medulla and A5 cell group project

directly to the dorsal horn of the spinal cord where transmission of nociceptive information is inhibited (Dampney, 1994; Sun, 1995; see Fig. 3). In addition, a projection between the VTA/SN and the PAGvl/DR may also influence neurons in the PAGvl that are involved in the regulation of nociception.

4.3 Functional Considerations

4.3.1 Role of the PAGvl in Nociception and Cardiovascular Regulation

It is well known that the PAG is a key component of the brain's descending pain modulatory circuit (Basbaum and Fields, 1984; Behbehani, 1995; Fields *et al.*, 1991). Activation of neurons in all areas of the PAG by a variety of methods, including electrical stimulation and injections of excitatory amino acids, produces antinociception and inhibition of nociceptive neurons in the spinal cord. Furthermore, microinjection of morphine into the PAGvl produces potent antinociception (Yaksh *et al.*, 1976; Bennett and Mayer, 1979). In fact, the PAGvl is a key site in the brain where systemic morphine is believed to produce its analgesic effect (Basbaum and Fields, 1984; Behbehani, 1995; Fields *et al.*, 1991). Electrophysiological experiments have shown that a proportion of neurons in the PAGvl respond to noxious or nociceptive stimuli applied to the body surface (Eickhoff *et al.*, 1978; Sanders *et al.*, 1980; Nakahama *et al.*, 1981).

This is also supported by studies using c-*fos* expression as an indication of neuronal activation, which show that noxious stimulations of deep tissue and viscera activate neurons in the PAGvl (Clement *et al.*, 1996; Keay and Bandler, 1993; Keay *et al*, 1994). In summary, nociceptive neurons in the PAGvl are believed to be part of a negative feedback system in which nociceptive stimuli activate the PAGvl circuitry involved in the production of antinociception.

A large amount of literature has accumulated on the role of the PAG in regulating emotional expression and cardiovascular function (Bandler *et al.*, 1991; Carrive and Bandler, 1991; Bandler and Shipley, 1994; Dampney, 1994; Behbehani, 1995; Sun, 1995). As presented in the introduction, PAG is arranged in functional columns. Activation of the PAGvl with electrical and chemical stimulation produces hypotension and bradycardia (Lovick, 1993; Bandler and Shipley, 1994). Neurons in the PAGvl are activated by decreases in blood pressure indicating that this area is involved in the regulation of the baroreceptor reflexes (Li and Dampney, 1995; Murphy *et al.*, 1995; Tassoreli and Joseph, 1995). Keay and Bandler (1998) have hypothesized that the PAGvl is the region of the brain where neurons are excited by deep somatic and visceral nociceptive inputs and may be involved in the production of cardiovascular responses to pain (Bandler and Shipley, 1994). Therefore, previous experiments suggested that the PAGvl is a critical brain region in which deep pain and haemorrhage inputs may be integrated to produce hypotension and bradycardia.

4.3.2 VTA/SN Regulation of the PAGvl/DR

This study describes a GABAergic projection between the VTA/SN and the PAGvl/DR. It is recognized that GABAergic neurons in the VTA project to other regions of the brain and do not function exclusively as interneurons within the VTA (Carr and Sesack, 2000; Steffensen *et al.*, 1998). In fact, a population of GABAergic neurons in the VTA appears to play a significant role in the rewarding properties of electrical brain self-stimulation (Steffensen *et al.*, 2001) and may project to forebrain sites through the internal capsule (Steffensen *et al.*, 1998). Therefore, it is likely that GABA projection neurons in the VTA or SN exert an influence on other sites in the brain including the PAGvl/DR.

A connection between the VTA/SN and the PAGvl/DR is of potential importance because of the numerous studies that support a role for the VTA and SN in the modulation of pain and arterial blood pressure, two functions regulated by the PAGvl/DR. However, a functional link between these midbrain regions has not received much consideration. Evidence for this functional link has been provided by the physiological experiments in this paper as well as previous observations (Kirouac and Pittman, 2000) showing that stimulation of the VTA/SN produces cardiovascular depressor responses that are mediated by a pathway to the PAGvl/DR. There is also considerable evidence that the VTA and SN play a role in pain modulation (Jurna *et al.*, 1978; Barnes *et al.*, 1979; Baumeister and

Frye, 1986; Frye *et al.*, 1986; Baumeister *et al.*, 1987, 1988, 1989, 1990, 1993; Hebert *et al.*, 1990; Morgan and Franklin, 1990; Baumeister, 1991; Altier and Stewart, 1993, 1996, 1997, 1998). At the present, there is no evidence that antinociception elicited by activation of neurons in the VTA/SN is mediated by a projection to the PAGvl/DR. Considering the fact that a significant GABAergic projection exists between the VTA/SN and the PAGvl/DR and that GABA is an important regulator of PAG pain modulating mechanisms (Reichling and Basbaum, 1990; Williams and Beitz, 1990; Osborne *et al.*, 1996; Roychowdhury and Fields, 1996; Hall and Behbehani, 1998), it is plausible that a GABAergic connection between the VTA/SN and the PAGvl/DR plays an important role in the modulation of pain. In addition to their role in pain modulation, there are numerous studies demonstrating that the VTA/SN receives nociceptive information (Barasi, 1979; Tsai *et al.*, 1980; Tsai, 1989; Ma *et al.*, 1993; Gao *et al.*, 1996; Smith *et al.*, 1997; Ohtori *et al.*, 2000) suggesting that pain may activate neural circuitry in the VTA/SN, which may in turn modulate the transmission of pain. It is also possible that the VTA/SN region modulates neural mechanisms in the PAGvl/DR involved in the production of behavioral and autonomic responses to pain and stress (Lovick, 1993; Behbehani, 1995; Bandler and Shipley, 1994; Bandler et al., 2000) including changes in blood pressure (Kirouac and Pittman, 2000). In this way, the VTA/SN would serve as a regulator of the PAGvl/DR circuitry involved in responses to pain and stress including the modulation of blood pressure and pain transmission.

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