# ANATOMICAL CHARACTERIZATION OF SEROTONERGIC AND NONSEROTONERGIC PROJECTIONS FROM THE DORSAL RAPHE NUCLEUS TO THE VESTIBULAR NUCLEI

by

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Preclinical and clinical evidence indicates that the serotonergic system regulates processing in the vestibular nuclei and in pathways linking balance function with emotional responses and affect. Previous studies conducted in this laboratory demonstrated that the serotonergic innervation of the vestibular nuclei is derived largely from the dorsal raphe nucleus (DRN), and revealed that the DRN also sends a nonserotonergic projection to the vestibular nuclei. The purpose of these experiments was to characterize the organization of the serotonergic and nonserotonergic components of the DRN projection to the vestibular nuclei.

In Chapter 3, we describe retrograde tracing experiments that examined whether DRN cells send collateralized projections to the vestibular nuclei and central amygdaloid nucleus (CeA), regions involved in the clinical linkage between disorders of balance control and anxiety, and concluded that a subset of the serotonergic and nonserotonergic projections to the vestibular nuclei also project to CeA.

Chapter 4 describes experiments with the anterograde tracer biotinylated dextran amine (BDA) that identified the terminal distribution of DRN projections within the vestibular nuclei. This study revealed that DRN projections descending in the ventricular plexus and the medial longitudinal fasciculus terminate within distinct vestibular terminal fields.

In Chapter 5, BDA was used in combination with the serotonergic neurotoxin 5,7dihydroxytryptamine (5,7-DHT) to selectively anterogradely trace nonserotonergic DRN projections to the vestibular nuclei. These experiments demonstrated that nonserotonergic DRN projections descend exclusively within the ventricular plexus and terminate primarily within the periventricular aspect of the vestibular nuclei.

The purpose of the experiments in Chapter 6 was to map the distribution of serotonergic DRN terminals within the vestibular nuclei; 5,7-DHT was injected directly into DRN and silver staining was used to visualize the resulting pattern of terminal degeneration. It appears that projections from serotonergic DRN neurons terminate within both medial and lateral regions of the vestibular nuclei.

Based on these findings, we conclude that major differences exist in the course of descent and termination patterns of serotonergic and nonserotonergic DRN projections to the vestibular nuclei, indicating that serotonergic and nonserotonergic cells give rise to distinct DRN projection systems that may selectively modulate processing within specific functional domains of the vestibular nuclei.

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#### PREFACE

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#### **1.0 INTRODUCTION**

#### 1.1 CENTRAL SEROTONERGIC PATHWAYS

A number of early reports noted the presence of a vasoconstrictive substance in serum and defibrinated blood (Gaddum, 1936; Reid and Bick, 1942; Zucker, 1944), but it was not until the pioneering work of Rapport (Rapport et al., 1948; Rapport, 1949) and Erspamer (Erspamer and Asero, 1952) that this compound was isolated and identified as being serotonin (5-hydroxytryptamine, 5-HT). In 1953, Gaddum reported that the hallucinogen lysergic acid diethylamide (LSD) antagonized the contractile effect of 5-HT on the isolated uterine smooth muscle (Gaddum, 1953). That finding, coupled with reports that 5-HT is present in the brains of dogs, rabbits and rats (Twarog and Page, 1953), led Woolley and Shaw (Woolley and Shaw, 1954) to suggest that 5-HT is involved in mental processing and possibly in the pathogenesis of schizophrenia. The subsequent discovery that reserpine, a sedative with antipsychotic properties, caused massive depletion of 5-HT provided additional indication that the neurochemical was somehow involved in mental illness (Shore et al., 1955). Although many workers accepted that 5-HT played some role in brain function (see for example Erspamer, 1961), the fact that investigators lacked even a rudimentary understanding of the anatomy of brain serotonergic systems greatly hindered work to elucidate the function of 5-HT in the central nervous system.

The presence of 5-HT within specific cells in the mammalian central nervous system was first demonstrated using the Falck–Hillarp fluorescence histochemical method (Dahlström and Fuxe, 1964). In the Falck–Hillarp method, freeze-dried tissue is exposed to gaseous formaldehyde which converts biogenic monoamines to derivatives that are intensely fluorescent. These fluorophores can be

distinguished based on their specific spectral properties, and the 5-HT fluorophore has a characteristic yellow fluorescence. Cell bodies containing 5-HT are found primarily within the brainstem raphe nuclei, and are clustered in specific groups which were designated as B1 through B9 (Dahlström and Fuxe, 1964). Fluorescence histochemistry also revealed that 5-HT-containing axon terminals are widely distributed throughout the brain (Fuxe, 1965). Unfortunately, the use of the Falck–Hillarp method to visualize the serotonergic system has several inherent limitations, including rapid photodecomposition of the 5-HT fluorophore, limited sensitivity, and incomplete detection of fine caliber 5-HT-containing fibers. The eventual development of immunohistochemical techniques that could selectively, sensitively, and reliably detect 5-HT (Steinbusch et al., 1978) finally allowed the serotonergic system to be mapped in great anatomical detail (Lidov et al., 1980; Steinbusch, 1981; Steinbusch et al., 1981; Lidov and Molliver, 1982; Jacobs et al., 1984).

The serotonergic raphe nuclei have been divided into inferior (caudal) and superior (rostral) groups based on their anatomical location within the brainstem and the developmental period during which they appear (Lidov and Molliver, 1982). The caudal division includes nucleus raphe obscurus (ROb; group B2), nucleus raphe pallidus (RPa; group B1), and nucleus raphe magnus (RMg; group B3), which are located in the medulla and caudal pons; the rostral division includes the caudal linear nucleus (CLi; group B8), dorsal raphe nucleus (DRN; groups B6 and B7), median raphe nucleus (MRN; groups B8 and B5), and supralemniscal nucleus (SLN; group B9), located in the midbrain and rostral pons (reviewed by Jacobs and Azmitia, 1992).

The largest cluster of 5-HT-containing perikarya in the brain is found in the DRN, and more than half of all the serotonergic neurons present in the brain are located within this nucleus (Leger and Wiklund, 1982). The DRN is located in the central grey in the ventral part of the midbrain periaqueductal grey (PAG), and it extends caudally into the rostral pons. The DRN has several subdivisions, including the dorsomedial (DRNdm), ventromedial (DRNvm), and lateral (DRNl) cell groups (Steinbusch et al., 1981). The cytoarchitecture of the DRN is illustrated in Figure 1.1. The dorsomedial and ventromedial subdivisions are centered on the midline, with the former group located immediately ventral to the



#### Figure 1.1. Schematic diagrams illustrating the DRNdm, DRNvm and DRNl subdivisions of the DRN.

(A) Rostral DRN level. (B) Middle DRN level. (C) Caudal DRN level. (D) Photomicrograph of 5-HTimmunostained cells in the DRN at the level of panel B. Cells containing 5-HT are clustered in the ventromedial (vm), dorsomedial (dm) and lateral (lat) subdivisions. The location of the mlf is indicated. 5-HT immunostaining was performed in Long-Evans rats using established procedures (see Halberstadt and Balaban, 2003). Scale bar=200 µm. cerebral aqueduct and the latter group situated dorsal to and in between the medial longitudinal fasciculus (mlf). The two lateral groups (or wings) extend into the lateral PAG and are bilaterally symmetric. Although it has been argued that the intrafascicular region of DRN should be treated as a separate nucleus (Azmitia, 1987), most classification schemes include the intrafascicular component as part of DRNvm (reviewed by Baumgarten and Grozdanovic, 2000).

A large number of studies have examined the organization of DRN projections using anterograde tracing. The earliest anterograde tracing experiments were conducted using autoradiographic tracing with tritiated amino acids (Conrad et al., 1974; Bobillier et al., 1976; Halaris et al., 1976; Pierce et al., 1976; Azmitia and Segal, 1978; Moore et al., 1978). These studies revealed that DRN cells project widely throughout the central nervous system, including the telencephalon, diencephalon, brain stem, cerebellum, and spinal cord. Unfortunately, in many of those studies the tracer injection was not confined to the DRN, and therefore MRN efferents were also labeled. Furthermore, the anatomical resolution of the technique is limited, and there are interpretive problems associated with the use of autoradiographic tracing. For example, because silver grains are visualized in a layer of nuclear track emulsion above the tissue section, axon morphology cannot be identified and it is difficult to identify some terminal fields.

The drawbacks associated with autoradiographic anterograde tracing led to the introduction of *Phaseolus vulgaris*-leucoagglutinin (PHA-L). PHA-L is a sensitive anterograde tracer that produces labeling with detailed morphological and anatomical resolution (Gerfen and Sawchenko, 1984). The PHA-L tracing method has been used to visualize the ascending and descending projections of the DRN (Vertes, 1991; Sim and Joseph, 1993; Vertes and Kocsis, 1994; Morin and Meyer-Bernstein, 1999). These studies revealed that DRN neurons project widely throughout the neuraxis, innervating most of the structures in the forebrain, midbrain, and brainstem. The organization of DRN projections to cerebral cortex (Kosofsky and Molliver, 1987), nucleus submedius (Matsuzaki et al., 1993), substantia nigra (Corvaja et al., 1993), piriform cortex (Datiche et al., 1995), hypothalamic paraventricular nucleus (Larsen et al., 1996), suprachiasmatic nucleus (Meyer-Bernstein and Morin, 1996; Hay-Schmidt et al.,

2003), subcommissural organ (Mikkelsen et al., 1997), substantia innominata and nucleus basalis magnocellularis (Gasbarri et al., 1999), and central amygdaloid nucleus (Commons et al., 2003) has also been examined in detail using PHA-L tracing.

More recently, biotinylated dextran amine (BDA) was developed as an anterograde tracer (Veenman et al., 1992; Reiner et al., 2000). BDA, which produces extremely detailed labeling of axons and terminal fields, offers several advantages over tracing with PHA-L. Compared with BDA, PHA-L requires a longer survival time (~4 days for BDA versus 21 days for PHA-L), and the histochemical procedures required for visualization of BDA labeling are much simpler than the procedures used for PHA-L. Studies have used BDA to anterogradely trace DRN projections to mesencephalic trigeminal nucleus (Li et al., 2000), cochlear nucleus (Thompson and Thompson, 2001; Ye and Kim, 2001), somatosensory cortex (Simpson et al., 2003), and septum (Aznar et al., 2004).

The DRN also contains an appreciable number of nonserotonergic neurons, and it has been reported that only 25–50% of the total DRN neuronal population contains 5-HT (Moore, 1981*a*). DRN cells have been shown to contain a number of neuroactive substances, including dopamine (Descarries et al., 1986; Yoshida et al., 1989; Stratford and Wirtshafter, 1990), GABA (Nanopoulos et al., 1982; Stamp and Semba, 1995), excitatory amino acids (Clements et al., 1987; Kiss et al., 2002), nitric oxide synthase (Xu and Hokfelt, 1997; Simpson et al., 2003), corticotrophin-releasing factor (CRF) (Uryu et al., 1992; Champagne et al., 1998), vasoactive intestinal polypeptide (VIP) (Petit et al., 1995; Kozicz et al., 1998), and cholecystokinin (CCK) (van der Kooy et al., 1981). Thus, it has been extensively demonstrated that DRN cells are neurochemically heterogeneous.

Although many investigators have treated DRN projections as being exclusively serotonergic, there is a good deal of evidence indicating that targets of DRN innervation receive input from both serotonergic and nonserotonergic cells. Retrograde tracing studies reveal that the percentage of extrinsically-projecting DRN neurons which are 5-HT-positive varies depending upon the target region, with reported values ranging from 15% to >97% (O'Hearn and Molliver, 1984; Beitz et al., 1986; Ma et al., 1991; Petrov et al., 1992; Van Bockstaele et al., 1993; Datiche et al., 1995; Kirifides et al., 2001;

Halberstadt and Balaban, 2003; Kim et al., 2004). It is known that the supramammillary nucleus (Kiss et al., 2002) and the inferior colliculus (Schwarz and Schwarz, 1992) are targeted by projections arising from glutamatergic DRN cells. GABAergic DRN cells project to the posterior lateral hypothalamus (Ford et al., 1995) and to prefrontal cortex (Jankowski and Sesack, 2002). CRF-immunoreactive DRN cells are a source of projections to the paraventricular nucleus (PVN) of the hypothalamus (Champagne et al., 1998) and to the central amygdaloid nucleus (CeA) (Uryu et al., 1992); however, it has been reported that CRF and 5-HT are co-localized in some DRN cells (Commons et al., 2003). The bed nucleus of the stria terminalis receives input from VIP-positive DRN cells (Petit et al., 1995; Kozicz et al., 1998). Simpson and colleagues (Simpson et al., 2003) demonstrated that nonserotonergic DRN neurons projecting to thalamus express nicotinamide adenine dinucleotide phosphate diaphorase, a marker for nitric oxide synthase. Additionally, dopaminergic DRN neurons have been shown to project to prefrontal cortex, neostriatum, nucleus accumbens, lateral septum, amygdala, and hippocampus (Pohle et al., 1984; Descarries et al., 1986; Yoshida et al., 1989; Stratford and Wirtshafter, 1990; Hasue and Shammah-Lagnado, 2002). The combination of anterograde tracing and 5-HT-immunofluorescent staining has revealed that while only 5–20% of the anterogradely labeled DRN-neocortical axons are 5-HT-negative (Kosofsky and Molliver, 1987), the majority of DRN fibers innervating the septum are nonserotonergic (Aznar et al., 2004). Finally, although Aghajanian et al. (1978) were unable to identify putative nonserotonergic DRN units that could be activated antidromically by electrical stimulation of the ventral tegmentum, Sawyer and coworkers (Sawyer et al., 1985) were able to antidromically activate a number of putative nonserotonergic DRN units by stimulation of the neostriatum.

Evidence indicates that nonserotonergic projections from DRN exert a modulatory influence on target structures. Hajés-Korcsok and Sharp (2002) found that the ability of DRN electrical stimulation to increase extracellular levels of noradrenaline in the hippocampus is unaffected by destruction of central serotonergic projections with 5,7-dihydroxytryptamine (5,7-DHT), a selective serotonergic neurotoxin; this suggests that noradrenergic neurons in the locus coeruleus (LC) are excited by non-5-HT DRN

projections. There is also evidence that disinhibition of rhythmically firing nonserotonergic DRN neurons results in excitation of olivocerebellar neurons (Weiss and Pellet, 1982). This disinhibition mechanism may contribute to harmaline-evoked generalized tremor in rats.

#### **1.2 PHARMACOLOGY OF SEROTONIN RECEPTORS**

5-HT has a potent contractile effect upon smooth muscle, especially rat uterus and guinea pig ileum. The first indication that there are multiple 5-HT receptor subtypes came from studies conducted by Gaddum and Picarelli (1957). These workers reported that treatment with either dibenzyline or morphine alone could only partially block the effect of 5-HT on guinea pig ileum. However, in tissue exposed to dibenzyline for 30 min, morphine markedly antagonized 5-HT-induced contraction. Likewise, after exposure of the tissue to morphine, dibenzyline acted as a full 5-HT antagonist. These findings demonstrated that 5-HT was acting through two different receptor classes (type D and type M) to induce contraction of guinea pig ileum.

Soon after the development of radioreceptor techniques to demonstrate receptor binding, this methodology was applied to the investigation of 5-HT receptors. The first radioligands utilized were [<sup>3</sup>H]LSD and [<sup>3</sup>H]5-HT (Bennett and Snyder, 1975, 1976). Both of these radioligands bound to rat brain membranes with high-affinity in a reversible, saturable, and stereoselective manner, indicating that they were binding to specific recognition sites. However, these workers noted that while LSD could displace both [<sup>3</sup>H]LSD and [<sup>3</sup>H]5-HT binding with equal affinity, 5-HT antagonists had much higher affinity for sites labeled with [<sup>3</sup>H]LSD. After introduction of the dopamine antagonist [<sup>3</sup>H]spiperone as a radioligand, it was recognized that [<sup>3</sup>H]spiperone binds to 5-HT receptors that are distinct from the sites labeled by [<sup>3</sup>H]5-HT (Peroutka and Snyder, 1979). The sites labeled by [<sup>3</sup>H]5-HT and [<sup>3</sup>H]spiperone were designated as 5-HT<sub>1</sub> and 5-HT<sub>2</sub> receptors, respectively; furthermore, it appeared that [<sup>3</sup>H]LSD labeled both of these 5-HT receptors. The D receptor was eventually shown to be equivalent to the 5-HT<sub>2</sub>

receptor, whereas the M receptor was pharmacologically distinct from the 5-HT<sub>1</sub> receptor and was later classified by Bradley and coworkers (Bradley et al., 1986) as the 5-HT<sub>3</sub> receptor.

In the subsequent two decades, the synthesis of more selective serotonergic ligands, coupled with the cloning of 5-HT receptor genes, has revealed that there are (at least) seven classes of serotonergic receptors (5-HT<sub>1</sub> through 5-HT<sub>7</sub>), with many of these receptor classes containing more than one subtype (reviewed by Hoyer et al., 1994, 2002). Members of the 5-HT<sub>1</sub> class of receptors include the 5-HT<sub>1A</sub>, 5-HT<sub>1B</sub>, 5-HT<sub>1D</sub>, 5-HT<sub>1E</sub> and 5-HT<sub>1F</sub> receptor subtypes; these receptors are all negatively coupled to adenylate cyclase. The 5-HT<sub>2</sub> receptor class includes three subtypes:  $5-HT_{2A}$  (equivalent to what was known historically as the 5-HT<sub>2</sub> receptor),  $5-HT_{2B}$ , and  $5-HT_{2C}$  (formerly known as the  $5-HT_{1C}$  receptor) (Hoyer et al., 1994). These three  $5-HT_2$  subtypes activate the phosphoinositide hydrolysis signaling cascade.

#### **1.3 INFLUENCE OF SEROTONIN ON VESTIBULAR FUNCTION**

There is evidence that central serotonergic mechanisms regulate vestibular function. These contributions may be particularly germane to the observed associations between vestibular dysfunction, anxiety disorders (reviewed by Balaban and Thayer, 2001; Balaban, 2002) and migraine (reviewed by Marcus et al., 2005; Furman et al., 2005). The first indication comes from clinical reports that selective serotonin reuptake inhibitors (SSRIs) are efficacious in the treatment of dizziness and vertigo (Johnson, 1998; Staab et al., 2004). The SSRIs also improve balance function in patients with panic disorder (Perna et al., 2003), in patients with anxiety disorders (Simon et al., 2005), and in patients with major or minor psychiatric symptoms (Staab et al., 2002). It has also been shown that it is more difficult to induce motion sickness in patients with migrainous vertigo after treatment with the 5-HT agonist rizatriptan (Marcus and Furman, 2006). Conversely, depletion of 5-HT induced by administration of a tryptophan-free diet increases the ability of illusionary visual movement to provoke motion sickness in volunteer

subjects (Drummond, 2005). Additionally, the SSRIs fluoxetine and paroxetine reduce postural instability in anxious mice of the BALB/cByJ strain (Venault et al., 2001). Furthermore, there is a marked vestibular component to the *SSRI discontinuation syndrome* in some patients: dizziness, vertigo and incoordination, symptoms that are exacerbated by head and eye movements (Black et al., 1993; Coupland et al., 1996; Lejoyeux and Adès, 1997; Zajecka et al., 1997; Michelson et al., 2000; Bogetto et al., 2002). The provocation and exacerbation of these balance symptoms by head and eye movements is suggestive of serotonergic modulation at the level of peripheral and central vestibular pathways. There is also evidence that the incidence of SSRI withdrawal may be especially high in patients with panic disorder (Black et al., 1993).

The vestibular nuclei are a probable site for the 5-HT–vestibular interactions. It has been shown using *in vivo* microdialyis that 5-HT is present in the medial vestibular nucleus (MVN) (Inoue et al., 2002). The concentration of 5-HT in MVN was elevated by K<sup>+</sup>-induced depolarization in a Ca<sup>2+</sup>-sensitive manner, indicating that 5-HT was being released from synaptic terminals. Immunohistochemical staining has demonstrated that the vestibular nuclei are innervated by fibers that are immunoreactive for 5-HT (Steinbusch, 1981, 1991; Harvey et al., 1993) and the serotonin transporter (SERT) (Halberstadt and Balaban, 2003). The density of these fibers is especially high along the surface of the fourth ventricle in MVN and superior vestibular nucleus (SVN), and the innervation density declines in more lateral and caudal regions of the vestibular nuclear complex. The appearance of SERT-positive axons in MVN is illustrated in photomicrographs in Figure 1.2.

Anatomical tracing studies in this laboratory (Halberstadt and Balaban, 2003; Halberstadt et al., 2003) have revealed that these fibers arise from DRN—especially from the rostral aspect of DRNvm and from caudal raphe nuclei (ROb, RPa, and RMg). After injection of Fluoro-Gold into the vestibular nuclei of Long-Evans rats, the greatest numbers of cells in DRNvm and ROb were labeled retrogradely from injection sites involving the rostral pole of SVN and the medial aspect of MVN, with few cells resulting from injections located further laterally. Conversely, the greatest numbers of cells in the dorsomedial and lateral DRN subdivisions were labeled retrogradely by injection sites that involved the



# Figure 1.2. SERT-immunoreactive fibers in rostral MVN (A) and mid-level MVN (B) in transverse sections (35 µm) from Long-Evans rats.

Note the density of SERT-positive fibers is greatest along the border of the ventricle and declines more laterally. Note also in panel B that SERT-positive fibers in the ventricular plexus extend laterally into MVN. These sections were immunostained for SERT using established procedures (see Halberstadt and Balaban, 2003). The location of the fourth ventricle (4v) is also indicated. Scale bar=50  $\mu$ m.

rostral pole of MVN, lateral caudal SVN, and the dorsolateral aspect of lateral vestibular nucleus (LVN); few cells were labeled from more caudal injection sites. These results demonstrate that SVN, MVN, and LVN are targeted by DRN projections; conversely, few DRN neurons were retrogradely labeled by injection sites located in the inferior vestibular nucleus (IVN), indicating that this vestibular nucleus receives only a weak projection from DRN. By using a combination of retrograde tracing and 5-HT immunofluorescent staining, it was possible to demonstrate that slightly more than half of the DRN cells projecting to the vestibular nuclei are serotonergic (Halberstadt and Balaban, 2003). It was subsequently verified by other workers that the DRN projects to MVN in the golden hamster (Horowitz et al., 2005).

Electrical stimulation of the DRN evokes electrophysiological responses in the vestibular nuclei that are attenuated by 5-HT receptor antagonists (Kishimoto et al., 1991; Licata et al., 1995). Moreover, in a number of *in vivo* and *in vitro* electrophysiological studies, 5-HT and selective 5-HT receptor agonists have been shown to alter the firing activity of neurons located in SVN, MVN, and LVN (Licata et al., 1990, 1991, 1993*a*; Kishimoto et al., 1991, 1994; Johnston et al., 1993; Amano et al., 2001; Li Volsi et al., 2001; Jeong et al., 2003, 2005). Indeed, it has been extensively demonstrated, using quantitative autoradiography (Pazos and Palacios, 1985; Pazos et al., 1985; Fischette et al., 1987) and immunohistochemistry (Kia et al., 1996; Cornea-Hébert et al., 1999; Fay and Kubin, 2000; Balaban, 2002) that the vestibular nuclei contain 5-HT<sub>1A</sub>, 5-HT<sub>2C</sub>, and 5-HT<sub>2C</sub> receptors. Additionally, *in situ* hybridization histochemistry has confirmed that vestibular nucleus neurons express mRNA coding for 5-HT<sub>1A</sub>, 5-HT<sub>1B</sub>, 5-HT<sub>1Da</sub>, 5-HT<sub>1F</sub>, 5-HT<sub>2A</sub>, 5-HT<sub>2C</sub>, and 5-HT<sub>3</sub> receptors (Pompeiano et al., 1994; Bruinvels et al., 1995; Fornesca et al., 2001). Therefore, several lines of evidence argue that serotonergic (and possibly nonserotonergic) projections from the DRN modulate processing in the vestibular nuclei.

#### 1.4 BALANCE-ANXIETY LINKAGE

Extensive evidence demonstrates that vestibular dysfunction and anxiety disorders are interlinked. For example, there is an elevated incidence of anxiety and panic disorder in patients with vestibular dysfunction (Persoons et al., 2003; Pollak et al., 2003), and patients with panic disorder often display concomitant symptoms of balance dysfunction (Sklare et al., 1990; Hoffman et al., 1994; Jacob et al., 1996; Ramos et al., 1996; Perna et al., 2001). Indeed, in certain panic disorder patients, balance abnormalities may contribute to agoraphobic avoidance (Yardley et al., 1995; Jacob et al., 1996), and patients with agoraphobia and vestibular dysfunction show improvement after vestibular rehabilitation therapy (Jacob et al., 2001). Furthermore, it appears that symptoms of anxiety and vestibular dysfunction are co-exacerbating (Yardley et al., 1998; Yardley, 2000).

It has been proposed that the association between vestibular dysfunction and anxiety disorders is mediated by an ascending vestibulo-parabrachial nucleus (PBN) network which links central vestibular pathways with neural circuits involved in the expression of anxiety, emotion, and conditioned fear (Balaban and Thayer, 2001; Balaban, 2002). The PBN serves as an integration site for homeostatic afferent pathways, and is involved in interoception (Gauiau and Bernard, 2002; Craig, 2002, 2003). The PBN receives direct visceral sensory input from nucleus tractus solitarius (Fulwiler and Saper, 1984; Herbert et al., 1990), and somatic sensory input from lamina I and the trigeminal nucleus (Fiel and Herbert, 1995; Bernard et al., 1995; Craig, 1995; Dallel et al., 2004). The vestibular nucleus provides a dense ascending projection to the parabrachial region, including the Kölliker-Fuse, medial parabrachial and lateral parabrachial nuclei (Balaban, 1996; Porter and Balaban, 1997; Balaban and Porter, 1998), and these projections convey information regarding whole-body motion to the PBN (Balaban et al., 2002). The PBN, in turn, projects to the central amygdaloid nucleus (CeA), insular and infralimbic cortex, and the paraventricular nucleus (PVN) and other hypothalamic nuclei (Fulwiler and Saper, 1984; Kapp et al., 1989; Bernard et al., 1993; Krukoff et al., 1993; Bester et al., 1997; Pritchard et al., 2000), central regions involved in the generation of fear and anxiety and their emotional, affective, and physiological

manifestations. This ascending vestibulo-PBN pathway functions as an interface between vestibular, somatic, and visceral information processing (Balaban and Thayer, 2001; Balaban, 2002).

There is evidence that processing in the ascending vestibulo-PBN-forebrain pathway is heavily modulated by DRN input. Anterograde tracing studies have demonstrated that the DRN projects to PBN (Sim and Joseph, 1993; Vertes and Kocsis, 1994), PVN (Vertes, 1991; Sim and Joseph, 1993; Larsen et al., 1996; Morin and Meyer-Bernstein, 1999), CeA (Vertes, 1991; Sim and Joseph, 1993; Morin and Meyer-Bernstein, 1999; Commons et al., 2003), and the insular and infralimbic cortices (Vertes, 1991; Morin and Meyer-Bernstein, 1999). These findings have been confirmed using retrograde tracing (Imai et al., 1986; Seroogy and Fallon, 1989; Ma et al., 1991; Uryu et al., 1992; Petrov et al., 1992, 1994; Li et al., 1993; Champagne et al., 1998; Hasue and Shammah-Lagnado, 2002), and the projections were found to originate from both serotonergic and nonserotonergic neurons. Numerous retrograde tracing studies have reported that individual DRN neurons send axon collaterals to two or more functionally related target structures (van der Kooy and Kuypers, 1979; van der Kooy and Hattori, 1980; de Olmos & Heimer, 1980; Köhler and Steinbusch, 1982; Imai et al., 1986; Van Boeckstaele et al., 1993; Kirifides et al., 2001). This organizational feature holds true for both serotonergic and nonserotonergic DRN neurons. It has been hypothesized that individual DRN neurons, because of such collateralized projections, may simultaneously influence processing in the vestibular nuclei and in downstream regions (Balaban, 2002). Interestingly, components of the vestibulo-parabrachial network are already known to be targets of collateralized DRN projections. For example, DRN cells send branching collaterals to the lateral PBN and the PVN (Petrov et al., 1992), and Petrov and colleagues (Petrov et al., 1994) have reported that the DRN is a source of collateralized projections to the CeA and the PVN. Importantly, preliminary retrograde tracing studies in our laboratory indicate that individual DRN cells send collateralized projections to CeA and the vestibular nuclei (Halberstadt et al., 2001). Hence, the rostral DRN may exert a coordinated modulatory influence on neural activity in components of the ascending vestibuloparabrachial network via collateralized projections to vestibular nuclei, PBN, hypothalamus, and amygdala.

#### 2.0 STATEMENT OF PROBLEM

The major goals of these studies will be to:

a) determine whether serotonergic neurons in the DRN that project to the vestibular nuclei also project via axon collaterals to the central nucleus of the amygdala. Experiments employing a combination of dual retrograde tracing and 5-HT immunostaining will test the hypothesis that projections from serotonergic DRN cells are organized to co-modulate processing in the vestibular nuclei and the central amygdaloid nucleus, regions implicated in mediating the linkage between vestibular dysfunction and anxiety disorders.

**b)** examine the regional termination patterns of projections from the DRN to the vestibular nuclei. By examining the regional distribution of DRN projections within the vestibular nuclei, we will determine if the DRN projects most heavily to medial and rostral aspects of the vestibular nuclei, as indicated by retrograde tracing studies. This goal will be accomplished using the anterograde tracer biotinylated dextran amine (BDA).

c) determine if there are substantial differences in the termination patterns of the individual serotonergic and nonserotonergic components of the projection from DRN to the vestibular nuclei. These experiments will a) determine whether it is possible to selectively anterogradely trace nonserotonergic DRN projections, b) determine whether it is possible to selectively anterogradely trace serotonergic DRN projections, and c) determine whether nonserotonergic and serotonergic DRN projections to the vestibular nuclei are differentially organized to modulate processing in the vestibular nuclei. The goal of selectively tracing nonserotonergic DRN projections will be accomplished using BDA anterograde tracing in animals that were previously treated with the selective 5-HT neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) to

destroy serotonergic cell somata and axons. The goal of selectively tracing serotonergic DRN projections will be accomplished by first destroying serotonergic cell somata in the DRN by local microinjection of 5,7-DHT, and then using amino–cupric–silver staining to visualize the distribution of degenerating serotonergic terminals.

# 3.0 SEROTONERGIC AND NONSEROTONERGIC NEURONS IN THE DORSAL RAPHE NUCLEUS SEND COLLATERALIZED PROJECTIONS TO BOTH THE VESTIBULAR NUCLEI AND THE CENTRAL AMYGDALOID NUCLEUS

#### 3.1 ABSTRACT

Using a combination of double retrograde tracing and serotonin immunofluorescence staining, we examined whether individual serotonergic and nonserotonergic neurons in the dorsal raphe nucleus (DRN) are sources of collateralized axonal projections to vestibular nuclei and the central amygdaloid nucleus in the rat. Following unilateral injections of Diamidino Yellow into the vestibular nuclei and Fast Blue into the central amygdaloid nucleus, it was observed that approximately one-fourth of the DRN neurons projecting to the vestibular nuclei send axon collaterals to the central amygdaloid nucleus. Immunofluorescence staining for serotonin revealed that more than half of the DRN neurons from which these collateralized projections arise contain serotonergic DRN cells may act to co-modulate processing in the vestibular nuclei and the central amygdaloid nucleus, regions implicated in the generation of emotional and affective responses to real and perceived motion.

The figures and text of this chapter are based on: Halberstadt AL, Balaban CD (2006) Serotonergic and nonserotonergic neurons in the dorsal raphe nucleus send collateralized projections to both the vestibular nuclei and the central amygdaloid nucleus. *Neuroscience* 140:1067–1077.

#### **3.2 INTRODUCTION**

Over the last few years, there has been increased recognition of the comorbidity between disorders of balance control and anxiety (Balaban and Thayer, 2001; Furman and Jacob, 2001; Furman et al., 2005). It has been proposed that the association between vestibular dysfunction and anxiety disorders is mediated by a vestibulo-PBN network which links central vestibular pathways with neural circuits involved in the expression of anxiety, emotion, and conditioned fear (Balaban and Thayer, 2001; Balaban, 2002). As reviewed by Balaban and Porter (1998), the vestibular nucleus is the source of dense ascending projections to the parabrachial region (Kölliker-Fuse, medial parabrachial and lateral parabrachial nuclei). The PBN, in turn, projects to the CeA, insular and infralimbic cortex, and PVN (Fulwiler and Saper, 1984), central regions involved in the generation of fear and anxiety and their emotional, affective, and physiological manifestations. This ascending vestibulo-PBN pathway serves as an interface between vestibular, somatic, and visceral information processing (Balaban and Thayer, 2001; Balaban, 2002).

Clinical evidence has suggested that 5-HT mechanisms play a role in the regulation of vestibular function (Johnson, 1998; Perna et al., 2003; Staab et al., 2002, 2004; Simon et al., 2005; Drummond, 2005; Marcus and Furman, 2006). Immunohistochemical staining for 5-HT (Steinbusch, 1991) and for the serotonin transporter (SERT) (Halberstadt and Balaban, 2003) has confirmed that the vestibular nuclei are innervated by serotonergic fibers. Studies conducted in our laboratory (Halberstadt and Balaban, 2003) using the retrograde tracer Fluoro-Gold further demonstrated that these fibers arise from neurons located within DRN and the caudal raphe nuclei (ROb and RPa). The projection from DRN appears to include both serotonergic and nonserotonergic components. Additionally, anterograde tracing conducted by Luppi et al. (1995) and by Vertes and Kocsis (1994) revealed that axons originating from DRN cells terminate in the vestibular nuclei, but did not distinguish between serotonergic and non-serotonergic contributions.

Retrograde tracing studies have established the principle that individual raphe neurons often send collateralized projections to two or more functionally related target structures (van der Kooy and

Kuypers, 1979; van der Kooy and Hattori, 1980; de Olmos and Heimer, 1980; Köhler and Steinbusch, 1982; Imai et al., 1986; Petrov et al., 1992; Van Boeckstaele et al., 1993; Petrov et al., 1994; Kirifides et al., 2001). Hence, Balaban (2002) suggested that collateralized DRN projections are a potential mechanism to co-modulate vestibular processing and processing in downstream regions (e.g., CeA, PVN) related to the emotional significance of vestibular and visceral stimuli. The CeA plays an important role in the expression of fear and anxiety in rats and primates (LeDoux et al., 1988; Kalin et al., 2004), and mediates the autonomic and behavioral responses associated with conditioned fear (LeDoux et al., 1988). Furthermore, anterograde tracing (Vertes, 1991; Commons et al., 2003) and retrograde tracing (Li et al., 1990; Petrov et al., 1994) studies have shown that both serotonergic and nonserotonergic DRN neurons project to CeA. Therefore, this study used a combination of double retrograde tracing and 5-HT immunofluorescence staining to examine whether serotonergic and nonserotonergic neurons in the DRN send collateralized axonal projections to CeA and the vestibular nuclei.

#### 3.3 MATERIALS AND METHODS

#### 3.3.1 Animals.

All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh, which certifies compliance with National Institutes of Health and United States Department of Agriculture standards for humane animal utilization. All efforts were made to minimize the number of animals used and their suffering. Animals were housed individually in suspended caging at 22 °C with a 12-h light/dark cycle and *ad libitum* access to food and water.

#### **3.3.2** Surgical Procedures

Adult male Long-Evans rats (300–500 g; Charles River Laboratories, Wilmington, MA, USA) were anesthetized with a combination of ketamine (50 mg/kg, i.m.), xylazine (6 mg/kg, i.m.) and acepromazine (0.5 mg/kg, i.m.), and fixed in a stereotaxic apparatus using ear bars and a bite bar. Stereotaxically guided injections were made through burr holes in the dorsal surface of the cranium. A 1 µl Hamilton syringe (Hamilton, Reno, NV, USA) was used to deliver unilateral pressure microinjections of 100 nl of Fast Blue (FB, 3% in 0.5 M NaCl, Sigma, St. Louis, MO, USA) into the CeA and 100 nl of Diamidino Yellow (DY, 2% in 0.5 M NaCl, Sigma) into the vestibular nuclei. A small number of animals received microinjections of FB in the vestibular nuclei and DY in the CeA; however, FB injected into the vestibular nuclei often spread outside the borders of the nucleus. The burr hole was then closed with Gelfoam and the scalp incision sutured.

#### **3.3.3** Euthanasia, Fixation and Sectioning.

After a 4–5-day survival period, the animals were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and perfused transcardially with phosphate-buffered saline (PBS, 0.9% NaCl in 50 mM phosphate buffer, pH 7.3) followed by paraformaldehyde–lysine–periodate (PLP) fixative (McLean and Nakane, 1974). The brains were extracted from the skulls, cryoprotected for 48–72 h at 4 °C in 30% sucrose–PBS, and 40 µm sections were cut serially in the transverse plane on a freezing–sliding microtome. The sections were mounted on gelatin/chrome alum-subbed slides, then processed immediately for 5-HT immunofluorescence histochemistry.
### 3.3.4 5-HT Immunofluorescent Staining

After washing with PBS, sections containing the raphe nuclei were treated overnight with blocking buffer (PBS with 2% bovine serum albumin (BSA) and 0.2% Triton X-100) containing 5% nonfat dry milk. The slide-mounted sections were then rinsed with PBS, and incubated for 24 h in a rabbit polyclonal anti-5-HT antibody (DiaSorin Inc., Stillwater, MN, USA) diluted 1:2000 with blocking buffer. Following extensive washing with PBS, the sections were treated for 3.5 h with a tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) diluted 1:50 in PBS containing 2% BSA. The sections were rinsed with PBS (8  $\times$  5 min), air-dried overnight in the dark, cleared with xylene, and coverslipped with non-fluorescent DPX (Fluka, Milwaukee, WI, USA).

Immunofluorescent staining for 5-HT was performed on slide-mounted sections in order to facilitate the serial analysis of labeling. No difference in the number or distribution of 5-HT-positive DRN cells was observed when these immunofluorescently stained sections were compared with sections that had been stained free-floating for 5-HT immunohistochemically using previously published methods (Halberstadt and Balaban, 2003).

# 3.3.5 Analysis

Retrograde labeling was analyzed under epifluorescence illumination with an Olympus BH-2 microscope coupled to a Cohu CCD video camera with a Diagnostic Instruments 0.6× objective. The images were captured on a 486-based computer equipped with a Matrox IP-8/AT video processing board (Matrox Electronics Systems, Dorval, Quebec, Canada) running OPTIMUS software (Ver. 4.02, BioScan, Edmonds, WA, USA). For each section, the borders of the raphe nuclei were traced with a "mouse" coupled to the computer, and the locations of labeled raphe cells were charted. The data from individual sections were subsequently plotted onto a standard series of camera lucida drawings.

Digital images of DY, FB, and/or TRITC-labeled cells were prepared using a Nikon Eclipse E600N microscope equipped with a Spot RT Monochrome camera (Model 2.1.1, Diagnostic Instruments, Inc., Sterling Heights, MI). The images were captured on a Pentium-based computer running MetaMorph software (Ver. 6.1r4, Universal Imaging Corp., Downingstown, PA). Adobe Photoshop 6.0 was used for brightness and contrast adjustments and cropping.

# 3.3.6 Vestibular Nucleus Nomenclature

The nomenclature used by previous retrograde tracing studies conducted in this laboratory (Schuerger and Balaban, 1999; Balaban et al., 2000; Halberstadt and Balaban, 2003) to characterize the location of retrograde tracer injection sites within the vestibular nuclei was retained in the present study for purposes of comparison. This classification scheme distinguishes several divisions of the vestibular nuclear complex, including superior vestibular nucleus (SVN), medial vestibular nucleus (MVN), lateral vestibular nucleus (LVN) pars  $\alpha$  (LVN $\alpha$ ), LVN pars  $\beta$  (LVN $\beta$ ), LVN pars  $\gamma$  (LVN $\gamma$ ), inferior vestibular nucleus (IVN), and group y.

#### 3.4 **RESULTS**

#### 3.4.1 Tracer Injection Sites

Of the 20 animals that received unilateral microinjections of the retrograde tracers FB and DY, 7 had injection sites that were centered in both target regions with minimal involvement of adjacent structures. All of these animals had FB injections in the CeA and DY injections in the vestibular nuclei. The FB and DY injection sites displayed a dense center bounded by a peripheral "halo" region of labeled neuronal somata; both of these regions have been treated as part of the injection site for the purpose of charting.



#### Figure 3.1. Photomicrographs of the FB and DY injections sites from case T3.

Panel (A) shows the center of the FB injection site in the CeA, and panel (B) shows the center of the DY injection site in MVN. Scale bars=500 µm. (Abbreviations: BLA, basolateral amygdaloid nucleus; IM, intercalated amygdaloid nucleus, main part; IVN, inferior vestibular nucleus; LaDL, lateral amygdaloid nucleus, dorsolateral part; NTS, nucleus tractus solitarius; PrH, nucleus prepositus hypoglossi).



# Figure 3.2. Schematic representations of Fast Blue injection sites in the central amygdaloid nucleus and Diamidino Yellow injection sites in the vestibular nuclei in (A) case 2005-19, (B) case R6, and (C) case R9.

The locations of the stereotaxic transverse sections are indicated by their coordinates relative to bregma. These diagrams were adapted from Paxinos and Watson (1998). Scale bar=1000 µm. (Abbreviations: ACo, anterior cortical amygdaloid nucleus; AIP, agranular insular cortex, posterior part; AStr, amygdalostriatal transition area; B, basal nucleus (Meynert); BLA, basolateral amygdaloid nucleus, anterior part; BLP, basolateral amygdaloid nucleus, posterior part; BLV, basolateral amygdaloid nucleus, ventral part; BMA, basomedial amygdaloid nucleus, anterior part; BMP, basomedial amygdaloid nucleus, posterior part; BST, bed nucleus of the stria terminalis; BSTIA, bed nucleus of the stria terminalis, intraamygdaloid division; CeC, central amygdaloid nucleus, capsular part; CeL, central amygdaloid nucleus, lateral division; CeM, central amygdaloid nucleus, medial division; CxA, cortexamygdala transition zone; DEn, dorsal endopiriform nucleus; DI, dysgranular insular cortex; Ect, ectorhinal cortex; I, inferior vestibular nucleus; icp, inferior cerebellar peduncle; IM, intercalated amygdaloid nucleus, main part; IMG, amygdaloid intramedullary gray; IPAC, interstitial nucleus of the posterior limb of the anterior commissure;  $L_{\alpha}$ ,  $L_{\beta}$ ,  $L_{\gamma}$ , pars  $\alpha$ ,  $\beta$  and  $\gamma$ , respectively, of the lateral vestibular nucleus; LaDL, lateral amygdaloid nucleus, dorsolateral part; LaVL, lateral amygdaloid nucleus, ventrolateral part; LaVM, lateral amygdaloid nucleus, ventromedial part; LEnt, lateral entorhinal cortex; LGP, lateral globus pallidus; M, medial vestibular nucleus; MeAD, medial amygdaloid nucleus, anterodorsal part; MeAV, medial amygdaloid nucleus, anteroventral part; MePD, medial amygdaloid nucleus, posterodorsal part; MePV, medial amygdaloid nucleus, posteroventral part; ph, nucleus prepositus hypoglossi; Pir, piriform cortex; PLCo, posterolateral cortical amygdaloid nucleus (C2); PMCo, posteromedial cortical amygdaloid nucleus (C3); PRh, perirhinal cortex; S, superior vestibular nucleus; SI, substantia innominata; VEn, ventral endopiriform nucleus; y, group y).

Four representative injection cases are illustrated in Figures 3.1 and 3.2. The FB injection sites were centered on the medial CeA division (injection case T4), the lateral CeA division (injection case R9), or both the medial and lateral divisions (injection cases 2005-15, 2005-19, R6, T1 and T3). Although the injection sites tended to extend beyond the boundaries of the CeA, in only two of the cases (injection cases R6 and R9) was there extensive involvement of adjacent amygdaloid subnuclei (specifically the basolateral and basomedial nuclei). The DY injection sites were centered within different regions of the vestibular nuclear complex. The injection in case 2005-15 involved IVN and a small lateral section of caudal MVN. Case 2005-19 involved medial caudal SVN, a dorsomedial portion of caudal LVNα, dorsal

LVN $\beta$ , and part of the lateral region of rostral MVN. In cases T1 and T3, the injection site was localized to caudal MVN and extends slightly into the medial aspect of rostral MVN. In case T4, the injection site was localized to caudal MVN. Injection site R6 was situated within the dorsolateral part of caudal MVN, with the edge of the site extending slightly into IVN. The tracer injection in case R9 is similar to that of T1 and T3, but with more extensive involvement of the medial part of rostral MVN.

# 3.4.2 Retrograde Labeling in DRN

Neurons labeled retrogradely with DY had a characteristic perinuclear yellow-gold fluorescence when illuminated with broad-band ultraviolet light, while neurons labeled retrogradely with FB displayed an ice-blue fluorescence in the perikarya. Under epi-illumination with green light, 5-HT-immunopositive, TRITC-labeled neurons and neuropil displayed bright red or orange-red fluorescence. Examples of DRN neurons singly- or doubly-labeled with FB and/or DY are shown in Figure 3.3A, C, E; some of these retrogradely-labeled DRN cells were also found to be 5-HT-immunopositive (Figure 3.3B, D, F).

As shown in Table 3.1, greater numbers of DRN cells were labeled retrogradely from the injection into CeA than the injection into the vestibular nuclei in each rat. The CeA is relatively compact anatomically and has a restricted rostrocaudal extent, and all of the tracer injection sites revealed similar involvement of CeA (see Figures 3.1 and 3.2). By contrast, the boundaries of the vestibular nuclei extend over a large A-P region in the rhombencephalon; hence, the tracer injections were confined to only a small part of the vestibular nuclear complex. Therefore, the potential number of double labeled DRN cells is limited by total number of labeled cells from the vestibular nuclei.

### **3.4.2.1 DRN Cells Projecting to Vestibular Nuclei.**

After microinjection of DY into the vestibular nuclei, retrogradely labeled neurons were detected throughout rostrocaudal extent of the DRN (Figures 3.4A, 3.5A, 3.6A). The majority of labeled neurons were located within the middle third of DRN. Neurons labeled retrogradely from the vestibular nuclei



Figure 3.3. Fluorescence photomicrographs of retrogradely labeled neurons (A,C,D) and 5-HTimmunopositive neurons (B,D,F) in the dorsal raphe nucleus (DRN) after injection of Diamidino Yellow (DY) into the vestibular nuclei and Fast Blue (FB) into the central amygdaloid nucleus.

(A, B) Low-power photomicrographs showing the distribution of DY- and FB-filled cells (in A) and 5-HTimmunopositive cells (in B) in the same DRN section. The rectangles in panels A and B indicate the area shown enlarged in panels C and D, respectively. The medial longitudinal fasciculus (mlf) is also labeled. (C, D) Highpower photomicrographs showing the distribution of DY- and FB-filled cells (in C) and 5-HT-immunopositive cells (in D) in the same DRN section as panels A and B. The DRN neurons labeled retrogradely with FB are indicated with arrows, neurons labeled only with DY are indicated with arrowheads, and neurons labeled with both FB and DY are indicated with asterisks. (E, F) Higher-power photomicrographs of a different site showing DY- and FBfilled cells (in E) and 5-HT-immunopositive neurons (in F) in the same DRN section. (E) The DRN neurons labeled retrogradely with FB are indicated with arrows, neurons labeled only with DY are indicated with arrowheads, and neurons labeled with both FB and DY are indicated with asterisks. (F) Four of the seven retrogradely labeled DRN neurons are also 5-HT-immunopositive. Scale bars=200 µm in A and B, 100 µm in C and D, and 25 µm in E and F.

#### Table 3.1. Total number of retrogradely labeled cells in the DRN.

Cell counts represent the total number of serotonergic (5-HT+) and nonserotonergic (5-HT-) DRN neurons that were labeled retrogradely with FB alone (from the central amygdaloid nucleus), with DY alone (from the vestibular nuclei), or with both FB and DY.

	F	В	DY		FB & DY	
Injection Case	5-HT+	5-HT-	5-HT+	5-HT-	5-HT+	5-HT-
2005-15	65	22	14	14	10	4
2005-19	54	43	24	13	14	2
R6	140	64	43	32	13	12
R9	112	59	58	35	18	15
T1	80	57	45	28	20	10
T3	72	66	42	30	19	12
T4	76	30	46	33	14	6

were found in DRNdm, DRNvm, and DRNI; labeled cells were occasionally seen within the medial longitudinal fasciculus. Consistent with our Fluoro-Gold results (Halberstadt and Balaban, 2003), the numbers of labeled cells varied with the location of the injection site (Table 3.1). The greatest numbers of labeled cells in DRN were observed after injections sites involving MVN (cases R6, R9, T1, T3 and T4). Fewer labeled cells were observed from case 2005-19, an injection centered in the rostrolateral part of the vestibular nuclear complex. The injection involving IVN (case 2005-15) was associated with the sparsest DRN labeling.

Across the seven rats, between 56% and 72% of the DRN neurons labeled retrogradely from the vestibular nuclei were also 5-HT-immunoreactive. The 5-HT-immunopositive DY-labeled cells were intermingled with 5-HT-immunonegative DY-labeled cells (Figures 3.4A, 3.5A, 3.6A). No differences were observed in the distribution of DY-labeled DRN cells that were 5-HT-immunopositive versus cells that were 5-HT-immunonegative.



Figure 3.4. Chartings of retrograde labeling in the DRN after injection of Diamidino Yellow (DY) into the vestibular nuclei and Fast Blue (FB) into the central amygdaloid nucleus of rat 2005-19.

Closed and open circles ( $\bullet$ , $\bigcirc$ ) represent FB-labeled DRN cells with and without 5-HT-immunoreactivity, respectively. Closed and open squares ( $\blacksquare$ , $\bigcirc$ ) represent DY-labeled DRN cells with and without 5-HT-immunoreactivity, respectively. Closed and open diamonds ( $\bullet$ , $\diamondsuit$ ) represent neurons that are double (FB/DY)- and triple (FB/DY/5-HT)-labeled, respectively. Each symbol represents one cell body. Each level shown represents a composite of labeled cells observed from five transverse sections at or near the indicated rostrocaudal level (interaural +0.70, +1.00, +1.96). Ipsilateral labeling is displayed to the right side of each drawing. The locations of 5-HT-positive and 5-HT-negative DRN cells that were labeled with DY or with both DY and FB are plotted in (A); the locations of 5-HT-positive and 5-HT-negative DRN cells that were labeled singly with FB are shown in (B). (Abbreviations: DRNI, dorsal raphe nucleus, lateral subdivision; DRNm, dorsal raphe nucleus, medial subdivision; mlf, medial longitudinal fasciculus).



Figure 3.5. Chartings of retrograde labeling in the DRN after injection of Diamidino Yellow into the vestibular nuclei and Fast Blue into the central amygdaloid nucleus of rat R6. The format is identical to Figure 3.4.

# 3.4.2.2 DRN Cells Projecting to CeA

The majority of cells labeled retrogradely from CeA were found in sections from the caudal and intermediate levels of the DRN, with relatively fewer cells labeled in the rostral DRN (Figures 3.4B, 3.5B, 3.6B). The cells labeled from CeA were interspersed with cells labeled retrogradely from the vestibular nuclei, but cells projecting to CeA were more likely to be located in the lateral DRN subdivision or within the intrafascicular part of DRNvm. It should be noted that the heaviest retrograde labeling was seen in those cases (injection cases R6 and R9) in which the injection site involved not only CeA but also the basolateral and basomedial amygdaloid nuclei.

Between 52% and 75% of the FB-labeled DRN cells were also immunoreactive for 5-HT. As was the case for the neurons projecting to the vestibular nuclei, there was no detectable difference in the regional distributions of 5-HT-positive and 5-HT-negative DRN cells that that were labeled retrogradely from CeA.



Figure 3.6. Chartings of retrograde labeling in the DRN after injection of Diamidino Yellow into the vestibular nuclei and Fast Blue into the central amygdaloid nucleus of rat R9.

The format is identical to Figure 3.4.

### 3.4.2.3 DRN Cells Sending Collateralized Projections to CeA and Vestibular Nuclei

DRN neurons containing both retrograde labels were found in all seven of the injection cases (see Table 3.1). The double-labeled cells were distributed in all DRN subdivisions (Figures 3.4A, 3.5A, 3.6A), but they were more numerous in the medial subdivisions compared with DRNI. Furthermore, greater numbers of double-labeled neurons were found in the intermediate DRN compared with more caudal and rostral levels. More than half of the double-labeled DRN neurons were also 5-HT-positive (Table 3.1), ranging from 52% in case R6 to 88% in case 2005-19.

The proportion of double-labeled cells, represented as a proportion of all single and doublelabeled DRN cells from vestibular nucleus injection sites, is summarized in Table 3.2. In the average case, approximately one-fourth (28%) of all DRN cells that were labeled retrogradely from the vestibular nuclei were also labeled from the CeA. The proportions were similar for both 5-HT-immunopositive and 5-HT-immunonegative neurons. The 5-HT-immunopositive and 5-HT-immunonegative cells were interspersed in DRN.

#### 3.5 DISCUSSION

The results of this study provide the first demonstration that both serotonergic and nonserotonergic DRN neurons send collateralized axonal projections to the vestibular nuclei and to CeA. These collateralized neurons form a significant proportion of the DRN cells that project to the vestibular nuclei, comprising approximately one-fourth of the total population. Hence, they may be a specialization for co-modulation of information processing in the vestibular nuclei and the vestibulo-PBN-CeA pathway.

# Table 3.2. Proportion of DRN cells labeled from the vestibular nuclei that were also labeled retrogradely from the central amygdaloid nucleus.

These proportions were calculated from the data in Table 3.1 as the number of DY labeled cells (labeled from the vestibular nuclei) divided by the sum of the number of DY labeled and double labeled neurons. Separate calculations are shown for all DRN neurons, serotonin-positive (5-HT+) retrogradely labeled neurons and serotonin-negative (5-HT-) retrogradely labeled DRN neurons.

	Proportion Double-Labeled				
Injection Case	Total DRN	5-HT+ DRN	5-HT- DRN		
2005-15	0.33	0.42	0.22		
2005-19	0.30	0.37	0.13		
R6	0.25	0.23	0.29		
R9	0.26	0.24	0.30		
T1	0.29	0.31	0.26		
T3	0.30	0.31	0.29		
T4	0.20	0.23	0.15		

#### **3.5.1** DRN Projections to Vestibular Nuclei

The pattern of DRN retrograde labeling from the vestibular nuclei is consistent with our previous retrograde tracing study (Halberstadt and Balaban, 2003), which reported that the vestibular nuclei are targeted by a regionally selective projection from DRN. The greatest numbers of DRN neurons were labeled retrogradely after injections of Fluoro-Gold into the rostral and medial aspects of the vestibular nuclei, with fewer DRN cells labeled by injections located further caudally or laterally. The largest numbers of labeled DRN cells in the present study were associated with tracer injections involving MVN, especially when the injection site extended into the rostral part of MVN (case R9). Conversely, the injection case primarily involving the IVN (*i.e.*, case 2005-15) produced the fewest retrogradely-labeled DRN cells. The findings were parallel for 5-HT-immunopositive and 5-HT-immunonegative neurons. With respect to the serotonergic projections, these findings are also consistent with immunohistochemical data (Steinbusch, 1991; Halberstadt and Balaban, 2003) showing that the density of 5-HT-

immunoreactive and SERT-immunoreactive fibers is greatest along the border of the fourth ventricle (in the medial parts of MVN and SVN) and that it declines in more lateral regions of the vestibular nuclear complex.

#### 3.5.2 DRN Projections to CeA

Although the CeA is innervated densely by projections originating from DRN (Vertes, 1991; Commons et al., 2003), there is some controversy in the literature regarding whether this projection contains an appreciable serotonergic component. For example, Steinbusch (1981, 1984) has reported that CeA lacks 5-HT-immunoreactive terminals, and Li et al. (1991) reported that only ~20% of the DRN cells projecting to CeA are serotonergic. Conversely, Asan et al. (2005) found moderate levels of 5-HT-immunoreactive and SERT-immunoreactive fibers in the lateral capsular and medial CeA of rats and mice, and Ma and coworkers (Ma et al., 1991) reported that the majority of projections from the DRN to the amygdala are 5-HT-positive. Indeed, immunohistochemical work performed in our laboratory has revealed that CeA contains moderate levels of SERT-immunopositive and 5-HT-immunopositive fibers in Long-Evans rats (Halberstadt and Balaban, unpublished observations). Our present results also indicated that a majority (>60%) of the DRN cells innervating CeA are serotonergic. Hence, they support the contention that CeA receives a substantial serotonergic innervation originating from the dorsal raphe.

### 3.5.3 Projections Originate From Serotonergic and Nonserotonergic DRN Cells

These experiments indicated that similar proportions of 5-HT-immunopositive DRN cells project to the vestibular nuclei alone (mean: 64%), to CeA alone (mean: 60%), or to both structures by collaterals (mean: 64%). These findings suggest that the collateralized component should be regarded as a representative sample of the serotonergic and non-serotonergic projections to the two sites. These estimates are within the considerable range of reported proportions (15% to >97%) of extrinsically-

projecting DRN neurons which are 5-HT-positive (Beitz et al., 1986; Ma et al., 1991; Petrov et al., 1992; Van Bockstaele et al., 1993; Datiche et al., 1995; Kirifides et al., 2001). Although it is not known which transmitter substance(s) are present in the nonserotonergic DRN cells projecting to the vestibular nuclei and CeA, there are several possible candidates. DRN cells have been shown to contain a number of neuroactive substances, including dopamine (Yoshida et al., 1989; Stratford and Wirtshafter, 1990), gamma-aminobutyric acid (Nanopoulos et al., 1982; Stamp and Semba, 1995), excitatory amino acids (Clements et al., 1987; Kiss et al., 2002), and neuropeptides (Van der Kooy et al., 1981; Uryu et al., 1992; Petit et al., 1995; Kozicz et al., 1998). Projections from 5-HT-immunoreactive, substance Pimmunoreactive and [Leu]enkephalin-immunoreactive DRN cells to the CeA have been documented with retrograde tracing (Li et al., 1990). Unfortunately, it was not determined whether these three transmitters are expressed by distinct populations of CeA-projecting DRN cells, or alternatively, if substance P and [Leu]enkephalin coexist with 5-HT in serotonergic DRN cells. This is significant, as there is controversy regarding whether 5-HT and substance P are ever colocalized in individual DRN cells in rats (Magoul et al., 1986; Sergeyev et al., 1999). Furthermore, Hasue and Shammah-Lagnado (2002) reported that approximately one-third of the DRN neurons retrogradely labeled by injection of Fluoro-Gold into the CeA express immunoreactivity for tyrosine hydroxylase (TH); the presence of dopamine in these cells was confirmed using immunostaining. CCK perikarya in the DRN have also been shown to contribute substantially to the innervation of CeA (Seroogy & Fallon, 1989). It has been observed that THimmunoreactive DRN neurons often contain CCK (Seroogy et al., 1988); hence, some of the DRN cells projecting to the CeA may colocalize dopamine and CCK. Importantly, it appears that CCKimmunoreactivity and 5-HT-immunoreactivity are expressed by separate populations of DRN cells (Van der Kooy et al., 1981). Although CRF-immunoreactive DRN cells are also known to project to the CeA (Uryu et al., 1992), it appears that these cells are serotonergic (Commons et al., 2003).

# 3.5.4 Collateralized DRN Projections

We have found that approximately one fourth of the DRN neurons labeled retrogradely from the vestibular nuclei send collateralized axonal projections to the CeA and the vestibular nuclei. Previous studies have reported that 7-38% of the neurons in the DRN send collateralized axonal projections to CeA and PVN (Petrov et al., 1994), hippocampus and medial entorhinal area (Köhler and Steinbusch, 1982), prefrontal cortex and nucleus accumbens (Van Bockstaele et al., 1993), PVN and lateral PBN (Petrov et al., 1992), and to functionally related sites in the trigeminal somatosensory pathway (Kirifides et al., 2001). The proportion of collateralized DRN-vestibular nuclear projection cells that are serotonergic (52-88%, mean: 64%) also appears to be greater than the serotonergic proportion of DRN cells for these other collateralized pathways, For example, it was noted that approximately half of the DRN neurons that send collateralized axonal projections to CeA and PVN contain 5-HT (Petrov et al., 1994). Petrov and colleagues (Petrov et al., 1992) have reported that only 8% of the DRN cells projecting to both PVN and lateral PBN contain 5-HT. Finally, 21% of the DRN neurons that project via axon collaterals to prefrontal cortex and nucleus accumbens are also 5-HT-immunoreactive (Van Bockstaele et al., 1993). These findings all contribute to the hypothesis that the proportion of collateralized DRN projections to the vestibular nuclei and CeA is a specialization for serotonergic co-modulation of activity in the vestibulo-PBN-CeA pathway.

### 3.5.5 Functional Significance of Collateralized DRN Projections

The heterogeneous nature of the ascending pathways from DRN to the vestibulo-parabrachio-forebrain network argues that the organization of DRN projections is substantially more complex then was once believed, with several levels of organization existing simultaneously. Individual DRN neurons often send collateralized projections to multiple levels of a single neural pathway (*e.g.*, Allen and Cechetto, 1994;

Kirifides et al., 2001). It appears that certain DRN cells project to a number of functionally related structures, allowing coordinated modulation of processing throughout the network, whereas other DRN cells project to only a subset of target nuclei within a central pathway, or innervate only one particular site, allowing a regionally specific regulation of neuronal activity. Furthermore, the organization may be different for projections from serotonergic versus nonserotonergic DRN cells. In addition to the fact that 8% of the DRN neurons projecting to both PVN and lateral PBN are serotonergic, Petrov et al. (1992) also reported that although 15% of the DRN cells that project only to the PVN are 5-HT-positive, no serotonergic DRN cells were found that project only to the lateral PBN. Thus, the lateral PBN receives input from both serotonergic and nonserotonergic DRN cells that also send collaterals to PVN, whereas there are nonserotonergic but not serotonergic DRN cells that project to PBN but not to PVN.

The presence of collateralized DRN projections to CeA and the vestibular nuclear complex indicates that the activity of individual DRN neurons is capable of simultaneously modulating processing in these two projection regions. As was noted in Section 1.3, 5-HT influences the firing activity of cells in the vestibular nuclei (Johnson et al., 1993; Li Volsi et al., 1995, 2001; Amano et al., 2001; Jeong et al., 2003, 2005), and it has been proposed that 5-HT is involved in maintaining the background activity of neurons in the vestibular nuclei (Licata et al., 1993*a*, 1995). It was reported recently that the 5- $HT_{1A/1B/1D}$ agonist rizatriptan acts to reduce motion sickness susceptibility in patients with migrainous vertigo (Marcus et al., 2005; Marcus and Furman, 2006); this finding suggests that actions at 5-HT receptors may modulate the sensitivity of neurons in the vestibular nuclei to perceived motion. Interestingly, Macedo and colleagues (Macedo et al., 2002) have reported that 5-HT acts in CeA to decrease thresholds for expression of fear and aversive responses. Therefore, it is possible that collateralized DRN serotonergic projections to vestibular nuclei and CeA act to coordinate processing in the ascending vestibulo-PBN-CeA pathway to modulate the likelihood that vestibular stimulation will provoke an aversive response. Jacobs and Fornal (1993) have theorized that activation of 5-HT-releasing neurons may simultaneously facilitate motor output and inhibit sensory information processing during execution of motor programs. Given that the expression of fear and anxiety can be regarded as a form of motor output that reflects

interoception (Craig, 2002), the collateralized serotonergic projections from DRN to the vestibular nuclei and CeA are a potential mechanism linking sensitivity of balance and postural pathways to contexts that evoke fear and anxiety. It is easy to appreciate the utility of such a linkage for situations regarding defensive postures or avoidance behaviors (or maneuvers). In addition, it raises the possibility that dysregulation of these neurons represents another potential etiology for linked balance and anxiety disorders.

# 4.0 ANTEROGRADE TRACING OF PROJECTIONS FROM DORSAL RAPHE NUCLEUS TO THE VESTIBULAR NUCLEI

# 4.1 ABSTRACT

This study used the anterograde transport of biotinylated dextran amine (BDA) to identify the course and terminal distribution of projections from the dorsal raphe nucleus (DRN) to the vestibular nuclei. After iontophoretic injection of BDA into the medial and lateral regions of DRN, anterogradely labeled fibers descend within the medial longitudinal fasciculus and the ventricular fiber plexus to terminate within two discrete regions of the vestibular nuclear complex. One terminal field was located primarily ipsilateral to the injection site and involved rostrodorsal aspects of the vestibular nuclei, including superior vestibular nucleus and rostral portions of the medial vestibular nucleus (MVN) and lateral vestibular nucleus (LVN). The other terminal field involved caudoventral aspects of both ipsilateral and contralateral MVN and LVN and was less heavily innervated. These findings confirm that the vestibular nuclei are targeted by a regionally-selective projection from the DRN. The segregation of DRN terminals into anatomically distinct fields indicates that the DRN–vestibular nucleus projections are organized to selectively modulate processing within specific functional domains of the vestibular nuclear complex. In particular, these terminal fields may be organized to modulate vestibular regions involved in eye-related velocity storage, coordination of vestibular and affective responses, and the bilateral coordination of horizontal eye movement reflexes.

The figures and text of this chapter are based on: Halberstadt AL, Balaban CD (2006) Anterograde tracing of projections from the dorsal raphe nucleus to the vestibular nuclei [submitted to *Neuroscience*]. Portions of the text were expanded where appropriate.

#### 4.2 INTRODUCTION

The DRN is a prominent source of input to the vestibular nuclei, and electrical stimulation of the DRN alters the discharge of cells in the vestibular nuclei of rats (Licata et al., 1995) and cats (Kishimoto et al., 1991). We have previously demonstrated, using retrograde tracing (Halberstadt and Balaban, 2003), that DRN neurons project to the vestibular nuclei. After injection of Fluoro-Gold into the vestibular nuclei, the greatest numbers of cells in DRNvm were labeled retrogradely from injection sites involving the rostral pole of the superior vestibular nucleus (SVN) and the medial aspect of the medial vestibular nucleus (MVN), with few cells resulting from injections displaced laterally. By contrast, the greatest numbers of cells in DRNdm and DRNI were labeled retrogradely by injections within the rostral pole of MVN, lateral caudal SVN, and the dorsolateral aspect of the lateral vestibular nucleus (LVN); few cells were labeled from more caudal and medial injection sites. Unfortunately, the density of neurons retrogradely labeled from a region of the vestibular nuclei does not necessarily reflect the actual density of the terminal arborization found in the region. Although retrograde tracing methods can demonstrate how many neurons project to the tracer injection site, they do not reveal the morphology of the axons, the location (anatomical boundaries) of the terminal regions, and the density of the terminal innervation. Therefore, this study used anterograde tracing with biotinylated dextran amine (BDA) to examine the innervation of vestibular nuclei by projections from the DRN.

#### 4.3 MATERIALS AND METHODS

### 4.3.1 Animals

All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh, which certifies compliance with National Institutes of Health and United States Department of Agriculture standards for humane animal utilization. All efforts were made to minimize the number of animals used and their suffering. Pairs of animals were housed in suspended caging at 22 °C with a 12-h light/dark cycle and *ad libitum* access to food and water.

# 4.3.2 Surgical Procedures

Twenty-three adult male Long-Evans rats (250–300 g; Charles River Laboratories) were anesthetized using a mixture of ketamine (50 mg/kg, i.m.), xylazine (6 mg/kg, i.m.), and acepromazine (0.5 mg/kg, i.m.), and then fixed in a stereotaxic apparatus using ear bars and a bite bar. A burr hole was drilled in the cranium, and a solution of 7.5% BDA (10,000 MW; Molecular Probes, Eugene, OR, USA) in 10 mM phosphate-buffer containing 0.5 M NaCl, pH 7.0, was injected iontophoretically (4 µA positive pulsed square wave, 15 s duty cycle, 15 min) into DRN using glass micropipettes (~40 µm tip diameter). The micropipette was positioned at an angle 26° to the vertical plane, and aimed at the following stereotaxic coordinates: 1.0 mm posterior to the interaural line, 0 mm lateral, and 6.5 mm ventral to the skull surface, according to the atlas of Paxinos and Watson (1998). The burr hole was then closed with Gelfoam and the scalp incision sutured.

#### 4.3.3 Euthanasia, Fixation and Sectioning

After a 5 day survival period, rats were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.), and perfused transcardially with PBS followed by PLP fixative. The brains were extracted from the skulls, post-fixed overnight at 4 °C, cryoprotected for 48–72 h at 4 °C in 30% sucrose–PBS, and 35 µm sections were cut in the transverse plane on a freezing–sliding microtome.

#### 4.3.4 BDA Staining Procedure

A one-step avidin-biotin-peroxidase (ABC) procedure was used to visualize BDA labeling. Free-floating sections were washed with distilled water ( $3 \times 5$  min) and then treated with 0.9% H<sub>2</sub>O<sub>2</sub> in distilled water for 3 min. The sections were washed with PBS ( $4 \times 3$  min), and pre-incubated with 0.5% Triton X-100 in PBS for 1 h at room temperature. Following extensive washing with PBS ( $10 \times 3$  min), sections were incubated in ABC reagent (Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. After five washes with PBS, sections were rinsed with 50 mM Tris buffer (pH 7.2) for 10 min, and reacted with nickel-enhanced diaminobenzidine tetrahydrochloride (DAB) chromagen (5 g ammonium nickel(II) sulfate, 40 mg DAB, and 16.6 µl 30% H<sub>2</sub>O<sub>2</sub> in 200 ml 0.5 M sodium acetate buffer, pH 6.0). Sections were rinsed with PBS and mounted on gelatin/chrome alum-subbed slides. After air-drying overnight, the sections were dehydrated through a graded series of ethanol, cleared with xylene, and coverslipped with DPX Mountant.

### 4.3.5 5-HT Immunostaining Procedure

The source of this material was two Long-Evans rats that had served as control injections in a study examining the effect of targeted intra-DRN injections of 5,7-dihydroxytryptamine on amino–cupric–silver staining (see Chapter 6.0 for details). These animals were anesthetized with sodium pentobarbital (100

mg/kg i.p.) and perfused transcardially with rinse solution (0.4% sucrose, 0.8% NaCl, 0.4% dextrose and 0.023% CaCl2 in 67 mM cacodylate buffer, pH 7.2–7.4) followed by fixative (4% paraformaldehyde and 4% sucrose in 67 mM cacodylate buffer, pH 7.2–7.4). After a 24 h period of storage at 4 °C, the brains were extracted from the skulls, cryoprotected in perfusion fixative containing 30% sucrose at 4 °C for 5–7 days, and then shipped to NeuroScience Associates (Knoxville, TN, USA). At NeuroScience Associates, the brains were embedded in a solid gelatin matrix, sectioned (coronal plane) at 40 μm, sets of every sixth section collected, and one set of sections was 5-HT immunostained. Briefly, after treatment with hydrogen peroxide and then with blocking serum, the sections were incubated with (1) a 1:750,000 dilution of polyclonal rabbit anti-5-HT antibody (Immunostar, Hudson, WI, USA) for 24 h at 4 °C, (2) a 1:200 dilution of goat anti-rabbit biotinylated secondary antibody (Vector Laboratories) for 30 min, and (3) Vectastain ABC reagent for 1 h, with thorough rinses between steps. Sections were treated with DAB chromagen, rinsed, mounted on gelatin/chrome alum subbed slides.

# 4.3.6 Analysis

Digital images of BDA injection sites and anterograde labeling were prepared using a Nikon Eclipse E600N microscope equipped with a Spot RT Monochrome camera (Model 2.1.1, Diagnostic Instruments). The images were captured on a Pentium-based computer running MetaMorph software (Ver. 6.1r4, Universal Imaging Corp.). Adobe Photoshop 7.01 was used for brightness and contrast adjustments and cropping.

Camera lucida drawings were prepared using an Olympus BH-2 microscope ( $20 \times$  objective) equipped with a drawing tube. The full-size drawings were reduced by 50%, traced in India ink, and scanned at 600–800 dpi in grayscale mode.

# 4.4 RESULTS

# 4.4.1 Injection Sites

Of the 23 animals that received iontophoretic BDA injections, 6 had injection sites that were centered in the DRN with minimal involvement of adjacent structures. Examples of BDA injection sites are illustrated in Figure 4.1. The BDA injection sites displayed a dense central core of labeled neuronal somata and neuropil surrounded by a peripheral region containing scattered neuronal perikarya with dendrites that extend into the core. These sites were generally ovoid and relatively small, with the diameter of the central core typically measuring 200 µm in the mediolateral direction and extending for a similar distance rostrocaudally across transverse sections. With the exception of injection case 323B which was located in caudal DRN, all of the injection sites were centered within the intermediate part of DRN. The BDA injection sites were located within different subdivisions of the DRN, although they often spanned multiple subdivisions. The injection site in case 323A was located in DRNdm with some involvement of DRNvm (Figure 4.1A). Injection case 210C was confined within DRNI (Figure 4.1B). Case 323B was situated in the ventral part of DRNI with some spread into the mlf (not shown). Injection case 401B was localized to DRNvm and the intrafascicular region of the DRN (Figure 4.1C). The tracer injection in case 329B (not shown) was made into DRNvm and the ventral part of DRNI, with some encroachment into the mlf. Case 420C was similar to 329B except that there was much less involvement of DRNvm (Figure 4.1D).

Axons could be traced from the injection site to their terminations in the vestibular nuclei. The distribution of BDA-labeled fibers within five representative transverse sections spanning the rostrocaudal extent of the vestibular nuclei from cases 210C, 323A, 329B, 401B, and 420C is illustrated in Figures 4.2–4.6. Transport ipsilateral to the injection site is illustrated to the right side of each figure. These camera lucida drawings reveal that the distribution of fibers and terminal regions was consistent across cases. Although there was little difference in the overall pattern of labeling, the density of labeled

fibers did vary between cases, depending upon the location of the injection site. Thus, injection cases primarily involving the lateral DRN subdivision (210C, 323B, 329B, 420C) were associated with greater numbers of anterogradely labeled fibers than were cases primarily involving either the ventromedial DRN subdivision (401B) or the dorsomedial DRN subdivision (323A).



Figure 4.1. Photomicrographs of the BDA injection sites in the DRN in (A) case 323A, (B) case 210C, (C) case 401B, and (D) case 420C.

The location of the medial longitudinal fasciculus (mlf) is also indicated. Scale bars=200 µm.



# Figure 4.2. Distribution of anterogradely labeled fibers in a series of transverse sections spanning the rostrocaudal extent of the vestibular nuclei from case 323A.

BDA-labeled fibers are charted in camera lucida drawings of five progressively caudal sections (A–E) located at the indicated AP level (relative to bregma). Transport ipsilateral to the injection site is illustrated on the right side of the figure. (Abbreviations: DTN, dorsal tegmental nucleus; g7, genu of facial nerve; IVN, inferior vestibular nucleus; LC, locus coeruleus; LVN, lateral vestibular nucleus; mlf, medial longitudinal fasciculus; MVN, medial vestibular nucleus; NTS, nucleus tractus solitarius; PBN, parabrachial nucleus; PrH, nucleus prepositus hypoglossi; scp, superior cerebellar peduncle; sg, supragenual nucleus; SLC, nucleus subcoeruleus; SUT, supratrigeminal nucleus; SVN, superior vestibular nucleus; y, group y).



Figure 4.3. Distribution of anterogradely labeled fibers in a series of transverse sections spanning the rostrocaudal extent of the vestibular nuclei from case 210C. The format is identical to Figure 4.2.



Figure 4.4. Distribution of anterogradely labeled fibers in a series of transverse sections spanning the rostrocaudal extent of the vestibular nuclei from case 401B. The format is identical to Figure 4.2.



Figure 4.5. Distribution of anterogradely labeled fibers in a series of transverse sections spanning the rostrocaudal extent of the vestibular nuclei from case 420C. The format is identical to Figure 4.2.



Figure 4.6. Distribution of anterogradely labeled fibers in a series of transverse sections spanning the rostrocaudal extent of the vestibular nuclei from case 329B. The format is identical to Figure 4.2.

#### 4.4.2 Course of BDA-Labeled Fibers Descending to the Vestibular Nuclei

The labeled fibers descended from the DRN to the vestibular nuclear complex along two routes: (1) the mlf, and (2) the ventricular plexus (see Figure 4.2 through Figure 4.6). The labeled fibers in these pathways differed in caliber.

Many large-caliber labeled fibers descended from the DRN injection site within the ipsilateral mlf. Along the extent of the mlf pathway, two bundles of fibers turned abruptly in the transverse plane to terminate in the vestibular nuclear complex. These fibers were similar in appearance to 5-HT-immunopositive fibers that descend within the mlf and terminate within the vestibular nuclei (Figure 4.7A). The first group of fibers (Figure 4.7B) emerged ipsilaterally from the mlf between stereotaxic levels AP -9.8 and AP -10.1 (Figures 4.2A, 4.3A, 4.4A, 4.5A, 4.6A). As they exited the mlf, these axons formed a broad band of fibers that traveled ventral to the posterodorsal trigeminal nucleus and dorsal to the genu of the facial nucleus. These fibers gave rise to smaller caliber terminal ramifications within LC (Figure 4.7C). However, the vast majority of the large caliber axons continued caudally as well as laterally, contributing projections to the *rostrodorsal terminal region* within the vestibular nuclear complex.

The second group of fibers (Figure 4.7D) exited the mlf bilaterally between stereotaxic levels AP -10.6 and AP -11.1 (Figures 4.2D, 4.3D, 4.4D, 4.5D, 4.6D), with the contralaterally projecting axons following the same course as axons remaining on the ipsilateral side. As they emerged laterally from the mlf, a substantial portion of these fibers traveled immediately ventral to the genu of the facial nerve. They then turned dorsally into the vestibular nuclear complex and formed a plexus in the *caudoventral terminal region*. Other fibers also innervated this terminal field, but they followed a course dorsal to the genu of the facial nerve and ventral to the border of the fourth ventricle.



**Figure 4.7.** Photomicrographs of (A) 5-HT-immunoreactive fibers in the medial longitudinal fasciculus (mlf) at the level of the rostral pons, and (B–F) BDA-labeled fibers descending from DRN to the vestibular nuclei. (A) Numerous large caliber 5-HT-positive fibers emerged laterally from mlf and coursed toward caudal locus coeruleus (LC) and the vestibular nuclei. (B) Large caliber, BDA-labeled fibers descending from DRN within mlf. At this level of the rostral pons, labeled fibers emerged ipsilaterally from the mlf, eventually supplying fibers to caudal LC and to the rostrodorsal vestibular terminal region. The fourth ventricle (4v) is also indicated. (C) Large caliber labeled fibers coursing laterally from mlf gave rise to small caliber terminal ramifications within caudal LC. (D) Further caudally, large caliber fibers exited the mlf bilaterally, eventually supplying fibers to the caudoventral vestibular terminal region. (E) In caudal DRN, small caliber fibers coursed dorsolaterally in a region adjacent to the border of the cerebral aqueduct, supplying fibers to the ventricular plexus. (F) Fine labeled fibers descending from the DRN within the ventricular plexus bordering the fourth ventricle. Scale bars=100 µm in A and B, 50 µm in C, E and F, and 200 µm in D.

Fibers also descended to the vestibular nuclei through the ventricular plexus. Within caudal DRN, fine labeled axons coursed dorsolaterally from the dorsomedial subdivision of DRN into the periaqueductal gray, adjacent to the border of the cerebral aqueduct (Figure 4.7E). These fibers descended within the ventricular plexus (Figure 4.7F), gradually fanning out dorsally. Within the rostral pons, a number of very fine fibers emerged laterally from the ventricular plexus, innervating medial portions of both the rostrodorsal and the caudoventral terminal fields.

### 4.4.3 Morphology of Labeled Axons in the Vestibular Nuclei

The anterogradely labeled DRN axons innervating the vestibular nuclei were morphologically heterogeneous. The majority of BDA-labeled fibers in the vestibular nuclei were of fine- to medium-caliber (<1  $\mu$ m in diameter) and displayed pleomorphic varicosities (Figure 4.8A–D, F–H). These axons had varicosities that ranged in appearance from small, granular enlargements to larger (1-2  $\mu$ m diameter) fusiform thickenings. The spacing of the varicosities along these axons was variable and often irregular, with thinner-caliber axons tending to have a greater distance between varicosities than heavier caliber axons. There did not seem to be any relationship between the diameter of the intervaricose axon segments and the diameter of the varicosities, although the varicosities on medium-caliber fibers were often relatively elongated. A second class of axons was relatively heavy in caliber (1–3  $\mu$ m in diameter) and had few, if any, varicosities (Figure 4.8B, E). Fibers of this type were observed descending within the mlf, and were found coursing laterally between the mlf and the vestibular terminal fields, but were only found in the vestibular nuclear complex in small numbers.



Figure 4.8. Morphology of anterogradely labeled axons and terminals present within the vestibular nuclei.

(A) Panel showing a fine caliber varicose fiber (indicated by the filled arrow) and a medium caliber varicose fiber (indicated by the open arrow). (B) Panel showing heavy caliber varicose fibers (indicated by filled arrows) and a fine caliber varicose fiber (indicated by the open arrow). (C) A small caliber varicose fiber traveling along the wall of a blood vessel. (D) Panel showing a medium caliber fiber with small, granular varicosities. (E) Example of a heavy caliber nonvaricose axon. (F, G) Panels showing fine caliber varicose fibers. Note the fusiform appearance of the varicosities. (H) Example of a very fine caliber varicose fiber. Scale bar=20 µm.

### 4.4.4 Terminal Fields in the Vestibular Nuclei

Anterograde labeling in the vestibular nuclear complex was found in two different terminal fields: (1) a *rostrodorsal* region, and (2) a smaller *caudoventral* region.

# 4.4.4.1 Rostrodorsal vestibular terminal region.

The rostrodorsal region is a large terminal field that spans the rostral two-thirds of the vestibular nuclei. At its most rostral extent (see level B of Figures 4.2–4.6), this termination region was restricted to the ventral edge of the rostral pole of SVN. At level C in Figures 4.2–4.6 (AP -10.6), labeled varicositybearing fibers were found in a broad field extending from the dorsal portion of rostral MVN to the ventral aspect of caudal SVN and to rostral LVN. Further caudally, at level D in Figures 4.2–4.6 (AP -11.1), this terminal region encompassed the dorsal part of MVN and a middorsal swath of caudal LVN. A mixture of medium- and fine-caliber labeled fibers was observed in LVN (Figure 4.9A, B); these fibers often branched extensively. Many fine fibers were distributed densely in SVN (Figure 4.9C). In the medial aspect of MVN, very fine fibers were scattered along the border of the fourth ventricle within the supraependymal plexus and the subventricular plexus; these fibers often extended laterally into periventricular regions of MVN (Figure 4.9D, E). In lateral portions of MVN, there were relatively large numbers of medium-caliber axons, both branched and unbranched (Figure 4.9D, E). These axons gave rise to a plexus of fine fibers (Figure 4.9E, F).

#### 4.4.4.2 Caudoventral vestibular terminal region.

The caudoventral terminal field was restricted to the caudal two-thirds of the vestibular nuclei (Figures 4.2–4.6). A mixture of medium- and small-caliber axons, some of which were highly branched, was observed in LVN (Figure 4.10A) and the rostral portions of MVN within this terminal field. Further caudally in MVN, there were very few medium-caliber axons (Figure 4.10B). As in the case of the rostrodorsal region, axons in MVN within the caudoventral region were the source of a plexus of fine


# Figure 4.9. Photomicrographs of labeled fibers and terminals within the rostrodorsal vestibular terminal region.

(A) Anterograde labeling in LVN. Note the presence of both small caliber and medium caliber varicose fibers. (B) Anterogradely labeled plexus of fine fibers in LVN. (C) Axon terminals in SVN. (D) Low-power photomicrograph showing the distribution of anterograde labeling in MVN. (E) High-power photomicrograph of the area indicated by the rectangle in panel D. Two small caliber axons (indicated by arrows) course dorsolaterally within the periventricular region of MVN, and relatively thicker axons are located further laterally. (F) Enlargement of the area indicated by the rectangle in panel E, showing labeled terminals. Scale bars=100  $\mu$ m in A and D, 50  $\mu$ m in B, C and E, and 10  $\mu$ m in F.



# Figure 4.10. Photomicrographs of labeled fibers and terminals within the caudoventral vestibular terminal region.

(A) There was a mixture of medium and fine caliber varicose axons in LVN. (B) Anterogradely labeled fibers and terminals in MVN. (C) Anterograde labeling in IVN. Note that the labeling in IVN was very sparse. Scale bar=50  $\mu$ m.

fibers. Very fine fibers also extended laterally into periventricular MVN from the ventricular plexus. The innervation of IVN consisted primarily of small-caliber fibers (Figure 4.10C). The density of labeled axons and terminals was substantially lower in the caudoventral field than in the rostrodorsal region. Beginning rostrally (level C of Figures 4.2–4.6), a light to moderate density of fibers was present in the ventral aspect of MVN. Further caudally (level D of Figures 4.2–4.6), the termination region extended from the ventral half of MVN through the ventral aspect of caudal LVN, near its border with IVN. At this level, fibers extended occasionally into IVN, but axonal labeling in this nucleus was generally very sparse. Light to moderately dense innervation was observed in caudal MVN (level E of Figures 4.2–4.6). As was noted in more rostral regions, the fibers traveling in caudal MVN sometimes entered IVN, but generally very few fibers were labeled in IVN.

#### 4.5 DISCUSSION

# 4.5.1 Multiple Terminal Fields in the Vestibular Nuclei

The present investigation demonstrates that projections from the DRN innervate two terminal regions within the vestibular nuclear complex: a rostrodorsal field and a caudoventral terminal field. Although retrograde tracing studies have indicated that DRN is a source of projections targeting the vestibular nuclei (Bellomo et al., 1991; Halberstadt and Balaban, 2003; Chapter 3.0), previous anterograde tracing studies have provided evidence that DRN projects only diffusely to the vestibular nuclei (Pierce et al., 1976; Vertes and Kocsis 1994; Luppi et al. 1995). Pierce et al. (1976) reported light anterograde transport of tritiated proline from the DRN to the vestibular nuclei. Similarly, Luppi et al. (1995) found only a few

anterogradely labeled fibers in the periventricular part of MVN after injecting cholera toxin subunit B (CTb) into the ventromedial subdivision of DRN. Finally, Vertes and Kocsis (1994) injected PHA-L into restricted sites in DRN. Although their illustrations show clearly labeled axons in the mlf pathways to the rostrodorsal and caudoventral terminal regions, they reported that anterograde axonal labeling was light in MVN after tracer injections in caudal DRN and virtually absent in the vestibular nuclei from other injection sites. The present findings reveal that the vestibular nuclei are innervated more extensively by projections originating in DRN.

Differences in the sensitivity of anterograde tracer methods provide a likely explanation for the underestimation of the extent of DRN projections to the vestibular nuclei in other studies. Even though BDA and PHA-L often yield equivalent results (Wouterland and Jorritsma-Byham, 1993; Dolleman-Van der Weel et al., 1994), the pattern of labeling revealed by BDA is sometimes more extensive than that obtained with PHA-L (Brandt and Apkarian, 1992; Alipour et al., 1997), and it has also been suggested that PHA-L does not completely label fine processes (Morin and Meyer-Bernstein, 1999). The detection of the mlf pathway to the vestibular nuclei (large caliber fibers) by Vertes and Kocsis (1994), but not the smaller caliber fibers present in the vestibular terminal regions, is consistent with this perspective. Similarly, Vertes and Kocsis (1994) reported that the DRN does not project to cochlear nucleus in the rat, whereas anterogradely labeled fibers have been observed in the cochlear nucleus of cats after injection of BDA into the DRN (Thompson and Thompson, 2001). Although species differences might account for this disparity, retrograde studies performed in rats (Klepper and Herbert, 1991) and guinea pigs (Thompson et al., 1995) have confirmed that the DRN projects to the cochlear nucleus.

The efferent projections from DRN follow two main pathways to the vestibular nuclei, the first pathway via the mlf and the second within the ventricular and periventricular plexus. Indeed, the results of previous tracing studies indicate that the ventricular system is innervated by projections originating within the DRN. An early autoradiographic tracing study conducted in cats observed light to heavy labeling in supraependymal areas throughout the ventricular system after microinfusion of tritiated proline into DRN (Pierce et al., 1976). Anterograde tracing experiments using PHA-L (Larsen et al., 1996;

Mikkelsen et al., 1997) or CTb (Didier-Bazes et al., 1997) in rats have demonstrated that DRN projections innervate the ventricular ependyma. Likewise, several studies have shown that after injection of CTb into the third ventricle, the fourth ventricle, or the lateral ventricle, DRN neurons are retrogradely labeled (Larsen et al., 1996; Didier-Bazes et al., 1997; Mikkelsen et al., 1997; Simpson et al., 1998). In our anterograde tracing material, a contingent of BDA-labeled fibers exited the DRN dorsolaterally before sending fibers to the ventricular and periventricular plexus within the PAG. Similar findings were reported by Larsen et al (1996) who observed that PHA-L-labeled fibers coursing dorsolaterally from DRN into the PAG contribute projections to the ventricular plexus.

The demonstration of rostrodorsal and caudoventral DRN projection fields in the vestibular nuclei is a novel finding. Several observations support the interpretation that BDA-labeled axons within the vestibular nuclei are distributed in two distinct terminal fields. First, a thorough visual examination of sections containing the vestibular nuclei failed to reveal the presence of any fibers whose course could be traced between the two terminal divisions. Second, for injection sites not centered along the midline, substantially larger numbers of labeled fibers were observed in the ipsilateral rostrodorsal terminal region compared with the rostrodorsal terminal region located on the contralateral side. Conversely, labeling in the caudoventral terminal field was bilateral. Lastly, the fibers that supply the rostrodorsal and caudoventral terminal fields exited laterally from the mlf at different rostrocaudal levels (e.g., compare Figure 4.7B and Figure 4.7D). The BDA anterograde labeling data are generally consistent with the extensive distribution and innervation density of axons in the vestibular nuclei that are immunopositive for 5-HT (Steinbusch, 1981, 1991; Harvey et al., 1993) and/or SERT (Halberstadt and Balaban, 2003). However, distinct terminal regions have not been resolved with these methods. This feature of the data is potentially explained by (1) the general principle that there are both serotonergic and non-serotonergic efferents from the DRN (O'Hearn and Molliver 1984; Beitz et al. 1986, Ma et al. 1991, Von Bockstaele et al. 1993; Datiche et al. 1995; Kirifides et al. 2001; Halberstadt and Balaban 2003) and (2) serotonergic innervation of the vestibular nuclei originates from multiple sites (*i.e.*, not every serotonergic axon in the vestibular nuclei originates from DRN). Since it is clear that DRN contributes both serotonergic and

nonserotonergic projections to the vestibular nuclei (Halberstadt and Balaban, 2003, 2006), it is likely that 5-HT- and SERT-immunostaining alone would tend to underestimate the distribution of DRN projections (*i.e.*, not every DRN efferent is serotonergic). Further, we have shown previously that the inferior raphe nuclei—ROb, and to a lesser extent RMg and RPa—project to the vestibular nuclei (Halberstadt and Balaban, 2003). One interesting possibility raised by these data, though, is that there may be topographic differences in the relative innervation patterns of serotonergic and non-serotonergic DRN projections to the vestibular nuclei.

The topographic pattern of anterograde labeling of DRN axons in the vestibular nuclei is consistent with the results of retrograde tracing studies (Halberstadt and Balaban, 2003). For example, a comparison of the two studies shows that the Fluoro-Gold injection sites that produced significant retrograde labeling in DRN all involved the rostrodorsal or caudoventral terminal fields. Similarly, the two caudolateral Fluoro-Gold sites that produced negligible retrograde transport were centered lateral to the caudoventral terminal field. In addition, the finding that the rostrodorsal field receives a denser plexus of DRN terminals than the caudoventral terminal field is consistent with the earlier finding that Fluoro-Gold injections in the rostral aspect of the vestibular nuclei produced more retrogradely labeled DRN cells than injections in the caudal aspect of the vestibular nuclei (Halberstadt and Balaban, 2003). In fact, the heaviest retrograde labeling from the earlier study was associated with injection sites that involved both the rostrodorsal terminal field and the DRN axon bundle entering that field. Retrograde tracing with Fluoro-Gold also revealed that fewer DRN cells were labeled by injections in the lateral aspect of the vestibular nuclei aspect of MVN. This may reflect the fact that in addition to being targeted by DRN projections descending in the mlf pathway, MVN is innervated preferentially by DRN projections which descend within the ventricular plexus.

The fact that the distribution of anterograde labeling is consistent with the retrograde labeling data from our previous studies (Halberstadt and Balaban, 2003) indicates that fiber-of-passage uptake of BDA (Brandt and Apkarian, 1992; Warr and Beck, 1996) is unlikely to be a major confounding factor in our results. Indeed, there is evidence that BDA uptake into fibers of passage is minimal from

iontophoretic application sites (Alipour et al., 1997; Power and Mitrofanis, 2002; van Dongen et al., 2005). However, fibers of passage uptake appears to result primarily from axons damaged by the pipet or microsyringe used to make the injection (Reiner et al., 2000), which will occur for any injection method. Thus, it is impossible to preclude the possibility that fiber of passage uptake contributed to the anterograde labeling observed in the vestibular nuclei in this study. A second confounding factor associated with BDA tracing is the possibility of axonal collateral-collateral transport (Chen and Aston-Jones, 1998), a process that occurs when BDA undergoes retrograde axonal transport and is then transported anterogradely along the axon collaterals of the same neuron. However, we did not observe an appreciable number of retrogradely labeled neurons in brain regions such as the parabrachial nucleus or locus coeruleus that project to both DRN and the vestibular nuclei, indicating that collateral-collateral transport did not contribute to the labeling in the vestibular nuclei.

The current results and previous retrograde tracing results (Halberstadt and Balaban 2003; Chapter 3.0) indicate further that projections to the rostrodorsal and caudoventral terminal fields arise from the same subdivisions of DRN. Small injections of BDA at different sites in DRN produced anterograde axonal labeling in both terminal regions. Conversely, injections of Fluoro-Gold in either terminal region produced retrograde labeling in the ventromedial, lateral, dorsomedial and mlf subdivisions of DRN.

Both the rostrodorsal and caudoventral terminal regions receive strong ipsilateral projections from the DRN, which is consistent with other DRN efferent connections. For example, retrograde tracing studies have demonstrated that the majority of DRN neurons projecting to caudate-putamen (Van der Kooy, 1979), neocortex (O'Hearn and Molliver, 1984), the olfactory bulb (McLean and Shipley, 1987), prefrontal cortex (Van Bockstaele et al., 1993), nucleus accumbens (Van Bockstaele et al., 1993), piriform cortex (Datiche et al., 1995), and components of the trigeminal somatosensory pathway (Kirifides et al., 2001) are located ipsilateral to the tracer injection site. However, our previous retrograde tracing study noted that DRN projections to the vestibular nuclei are bilateral (Halberstadt and Balaban, 2003), with relatively symmetric numbers of cells contributing to the ipsilateral and contralateral projections. The anterograde tracing data further demonstrate that the rostrodorsal vestibular terminal field receives a relatively sparse and restricted projection from the contralateral DRN, whereas the caudoventral vestibular terminal field receives a much more substantial plexus of terminals originating in the contralateral DRN. This difference in elaboration of contralateral DRN terminals suggests that the rostrodorsal and caudoventral terminal regions may represent two distinct functional pathways.

# 4.5.2 Relationship of DRN Terminal Fields to Vestibular Nucleus Organization

The vestibular nuclei contain several functionally distinct pools of neurons which are distributed widely over an extended rostrocaudal area (reviewed by Büttner-Ennever, 1992). Furthermore, afferents to and efferents from the vestibular nuclei are organized into rostral and caudal groups. The fact that DRN terminals within the vestibular nuclei are segregated in anatomically distinct fields suggests that the DRN-vestibular nucleus projections are organized to selectively modulate processing within specific functional domains of the vestibular nuclear complex. The rostrodorsal DRN terminal region encompasses the location of neurons in SVN and the rostral part of medial MVN that receive input from Purkinje cells in the nodulus and ventral uvula (Xiong and Matsushita, 2000). Because the nodulus and uvula are implicated in controlling the time constant of the velocity storage integrator (Waespe et al., 1985; Wearne et al., 1998), the rostrodorsal DRN terminal field may influence uvula and nodulus modulation of the velocity storage mechanism in vestibulo-ocular reflex pathways. Additionally, the presence of reciprocal connections between the nodulus and the vestibular nuclei (Epema et al., 1990; Xiong and Matsushita, 2000) raises the possibility that the gating of vestibulocerebellar transmission by feedback from Purkinje cells may be regulated by DRN activity. It should be noted that regions of the caudal vestibular nuclei that are innervated by the caudoventral DRN terminal field are also heavily targeted by projections from the nodulus (Xiong and Matsushita, 2000). These findings lend further support to the concept that serotonergic innervation of the vestibular nuclei is consistent with its role as an accessory 'cerebellar nucleus' (see Halberstadt and Balaban, 2003). Serotonergic innervation from the DRN, ROb and RPa innervate both the cerebellar nuclei (Kitzman and Bishop, 1994) and vestibular nuclei (Vertes and Kocsis, 1994; Halberstadt and Balaban, 2003). By contrast, extraraphe serotonergic cell groups, including cells in nucleus reticularis gigantocellularis, nucleus reticularis paragigantocellularis, lateral reticular nucleus and the lateral tegmental field provide serotonergic innervation to cerebellar cortex (Bishop and Ho, 1985, Kerr and Bishop, 1991).

Anterograde and retrograde tracing experiments conducted in rats (Porter and Balaban, 1997) and rabbits (Balaban, 1996) have demonstrated that several regions of the parabrachial nuclear complex are targeted by projections originating from both a rostral field (SVN, the dorsal part of rostral MVN, and ventral LVN (LVN pars  $\alpha$ )) and a caudal field (IVN, caudal MVN, and ventral LVN) within the vestibular nuclei. The PBN sends projections to forebrain regions involved in the regulation of autonomic function, including CeA, insular and infralimbic cortex, and PVN (Fulwiler and Saper, 1984; Kapp et al., 1989; Bernard et al., 1993; Krukoff et al., 1993; Bester et al., 1997). The ascending vestibulo-PBN-forebrain network serves to link vestibular pathways with central regions implicated in the expression of anxiety. avoidance conditioning, and conditioned fear (Balaban and Thayer, 2001; Balaban, 2002; Furman et al. 2005), and this pathway may be responsible for the association between vertigo and anxiety disorders. The fact that DRN cells send branching collaterals to the vestibular nuclei and CeA (Chapter 3.0), to lateral PBN and PVN (Petrov et al., 1992), and to CeA and PVN (Petrov et al., 1994), indicates that the DRN may exert a coordinated modulatory influence on neuronal activity in components of the vestibulo-PBN-forebrain pathway via collateralized projections. Interestingly, the same parts of the caudal vestibular nuclei that target the PBN also project heavily to sympathetic and parasympathetic brainstem regions, including nucleus tractus solitarius, dorsal motor vagal nucleus, nucleus ambiguus, and the ventrolateral medulla (Balaban and Beryozkin, 1994; Balaban, 1996; Porter and Balaban, 1997). Given that the rostrodorsal DRN terminal region heavily involves SVN, the dorsal part of rostral MVN, and the dorsal but not the ventral aspect of LVN, this DRN terminal region may influence the vestibuloautonomic regions that project to the PBN but not to brainstem autonomic nuclei. Conversely, the regions of the caudal vestibular nuclei that project heavily to PBN and the brainstem autonomic nucleiIVN and the ventral part of caudal MVN (Porter and Balaban, 1997)—are only weakly targeted by the caudoventral DRN terminal field, indicating that this DRN terminal region may have little involvement in modulating vestibulo-autonomic processing.

The caudoventral region, by contrast, appears to be related primarily to the caudal half of the MVN. Using two antigenically distinct recombinant strains of pseudorabies virus, Billig and Balaban (2005) examined whether neurons in the vestibular nucleus send collateralized projections to oculomotor pathways targeting motoneuron pools that control the lateral rectus muscle of one eye and the medial rectus muscle of the opposite eye. Cells in the vestibular nuclei containing dual viral labeling were localized primarily within the caudal two-thirds of the parvocellular part of MVN, suggesting that this population of neurons participates in the control of conjugate horizontal eye movements. Given that the caudoventral vestibular terminal field receives bilateral innervation from the DRN, it is possible that the DRN projection to this vestibular terminal region is specifically organized to exert a modulatory influence upon conjugate angular and/or linear vestibulo-ocular reflex eye movements. The magnocellular MVN has also been shown to provide extensive commissural fiber connections to contralateral MVN (Carleton and Carpenter, 1983; Ito et al., 1985; Newlands et al., 1989). The presence of these commissural connections allows the vestibular nuclei to operate in an integrated bilateral manner in response to input from primary vestibular afferents, which are exclusively ipsilateral in nature. The contribution of both ipsilateral and contralateral DRN to the caudoventral terminal field may allow for coordinated modulation of vestibular processing on both sides of the brain by input from the DRN.

# 4.5.3 DRN Projections to Locus Coeruleus and the Coeruleo-Vestibular Pathway

The anterogradely labeled fibers and terminals in the caudal LC after injection of BDA into the DRN are consistent with a spatially segregated DRN–LC pathway. Although two studies failed to observe labeling of DRN cells after injection of retrograde tracers into LC (Aston-Jones et al., 1986; Pieribone et al., 1988), a number of other retrograde tracing studies have shown that the LC is targeted by projections

originating from DRN (Sakai et al., 1977; Cedarbaum and Aghajanian, 1978; Morgane and Jacobs, 1979; Imai et al., 1986; Luppi et al., 1995; Kim et al., 2004). Indeed, Vertes and Kocsis (1994) reported that large numbers of fibers in LC were labeled anterogradely after injection of PHA-L into rostral or caudal DRN. Sim and Joseph (1993) also observed anterograde labeling in the LC after injection of PHA-L into DRN, but they did not describe the density of this innervation. Interestingly, we found that the ventral part of caudal LC is densely innervated by the same DRN projection pathway that contributes to the rostrodorsal terminal field within the vestibular nuclear complex. This observation is significant because the same region of LC is the source of the coeruleo-vestibular pathway (Schuerger and Balaban, 1993, 1999), a projection that is a major source of noradrenergic input to the vestibular nuclei. Schuerger and Balaban (1993, 1999) reported that the distribution of dopamine  $\beta$ -hydroxylase-immunoreactive (*i.e.*, noradrenergic) fibers in the vestibular nuclei of rats is regionally selective. The density of putative noradrenergic fibers is greatest in the dorsal LVN (LVN pars  $\gamma$ ) and the rostral part of ventral LVN (LVN pars  $\alpha$ ); an intermediate density of noradrenergic innervation was located in SVN and the remaining parts of LVN. Conversely, MVN received only a weak noradrenergic innervation, and the innervation of IVN was extremely sparse. The present findings demonstrate that some of the regions of the vestibular nuclei that receive the densest noradrenergic plexus—SVN and dorsal LVN—are also heavily innervated by the rostrodorsal DRN terminal field.

# 4.5.4 Morphology of DRN Projections Innervating the Vestibular Nuclei

Based on a morphological analysis of PHA-L anterograde labeling within rat cerebral cortex, Kosofsky and Molliver (1987) reported that the DRN gives rise to axons that are generally of fine caliber and have small fusiform or granular varicosities (type D axons). Conversely, they reported that axons arising from MRN are generally thicker and have large, spherical varicosities, and are characterized by a beaded appearance (type M axons). They further reported that type D and M axons have different laminar and regional distributions in the central nervous system. Immunocytochemical analysis of 5-HT-

immunostaining in cortex revealed the presence of two corresponding morphologic classes of axons. Subsequent studies have revealed that the same two distinct classes of 5-HT-immunoreactive fibers are present in the neocortex and hippocampus of the macaque monkey (Wilson et al., 1989) and the marmoset (Hornung et al., 1990). Similar findings have also been reported for cats (Mulligan and Tork, 1988; Leger et al., 2001) and ferrets (Voigt and de Lima, 1991). Further support for the differentiation of type D and type M axons is derived from reports that these classes of axons are differentially vulnerable to the neurotoxic effects of ring-substituted amphetamine derivatives (Mamounas and Molliver, 1988; O'Hearn et al., 1988; Mamounas et al., 1991; Harvey et al., 1993; Brown and Molliver, 2000). Treatment with 3,4-methylenedioxyamphetamine (MDA), *N*-methyl-MDA (MDMA), or *p*-chloroamphetamine (PCA) caused a profound loss of fine 5-HT-positive axons, whereas axons with large, spherical varicosities were spared.

Other investigators have reported findings that contradict the view that DRN and MRN projections can be distinguished solely on the basis of terminal size and shape. For example, in a PHA-L study of ascending DRN projections in the hamster, Morin and Meyer-Bernstein (1999) noted the occasional presence of beaded fibers in cortex, hippocampus and septum. Furthermore, it has been reported that after PHA-L injection into the DRN of rats, a mixture of fine fibers with small varicosities and thick fibers with large varicosities are labeled in substantia nigra (Corvaja et al., 1993). Workers have also shown in the rat that the MRN gives rise to both coarse and fine fibers that innervate the hippocampus (Halasy et al., 1992).

Our anterograde tracing experiments labeled DRN axons with both type D and type M-like morphologies after injections of BDA into the DRN. One population of axons had granular or fusiform varicosities which is a characteristic of type D axons. The vast majority of labeled fibers within the vestibular nuclei belonged to this class of fibers; this finding is consistent with the contention of Molliver and colleagues that type D axons arise from DRN. Importantly, we did not detect axons having large spherical varicosities in the vestibular nuclei after injection of tracer into DRN. It is important to note that while the cortex is innervated by type D fibers that are extremely fine in nature (Kosofsky and Molliver, 1987), many of the type D fibers that innervate the vestibular nuclei are of comparatively

heavier caliber. Thus, it would appear that axons arising from DRN may exhibit a greater degree of morphological variation than is indicated by the findings of Kosofsky and Molliver (1987). Indeed, it has been proposed that the exact morphology of DRN projections may vary depending on the target region (Morin and Meyer-Bernstein, 1999).

The second type of axon observed in vestibular nuclei was relatively thick and typically nonvaricose. These heavy-caliber fibers, which most likely represent axons of passage, bear resemblance to the non-varicose axons which have been noted by a number of 5-HT immunostaining studies (Lidov et al., 1980; Mulligan and Törk, 1988; Leger et al., 2001). Mulligan and Törk (1988) have suggested, based on immunocytochemical evidence from cat cortex, that the 5-HT-positive non-varicose axons are the parent axons of fibers with large, round varicosities—*i.e.* type M axons. However, this conclusion is clearly inconsistent with the present finding that large non-varicose fibers are labeled with BDA after injection of the tracer into DRN. This discrepancy provides additional support for the argument that the morphology of DRN and MRN axonal projections is target-specific.

#### 4.5.5 Interpretation of These Findings

This study has demonstrated that the innervation of the vestibular nuclei by projections from DRN is regionally specific. The rostrodorsal terminal field is organized to modulate vestibular regions involved in velocity storage and the coordination of vestibular and affective responses, whereas the caudoventral terminal field may be primarily involved in the bilateral coordination of vestibular-ocular reflexes. The firing activity of putative serotonergic DRN units in behaving cats is strongly tied to the sleep–wake– arousal cycle (Trulson and Jacobs, 1979; Sakai and Crochet, 2001) and is related to the level of tonic motor activity (Trulson et al., 1981; Steinfels et al., 1983). Thus, serotonergic input from the DRN may act to coordinate activity in the vestibular nuclei with the level of behavioral activation and tonic motor output. However, the DRN also contains a substantial number of nonserotonergic neurons, some of which project to the vestibular nuclei (Halberstadt and Balaban, 2003; Chapter 3.0). There appear to be

differences in the activity and firing properties of putative serotonergic and nonserotonergic DRN units (Aghajanian et al., 1978; Sakai and Crochet, 2001; Waterhouse et al., 2004). The ultimate influence of DRN afferents on neuronal activity within particular regions of the vestibular nuclear complex is therefore likely to depend, at least to some extent, on the local organization and termination patterns of the individual serotonergic and nonserotonergic DRN projections. Although Kosofsky and Molliver (1987) reported that 5-HT-negative anterogradely labeled fibers are often found interposed with 5-HT-positive anterogradely labeled fibers in cerebral cortex after injection of PHA-L into the DRN, it is not known whether the distribution of serotonergic and nonserotonergic DRN projections in other target regions is similar. The present study demonstrates that the vestibular nuclei are innervated by projections from the DRN, but interpretation of these findings needs to be taken with caution because differences may exist in the termination patterns of the individual serotonergic components of this pathway.

# 5.0 SELECTIVE ANTEROGRADE TRACING OF NONSEROTONERGIC PROJECTIONS FROM THE DORSAL RAPHE NUCLEUS TO THE VESTIBULAR NUCLEI

# 5.1 ABSTRACT

This study examined the termination patterns of nonserotonergic projections from DRN to the vestibular nuclei. Nonserotonergic DRN projections were selectively anterogradely traced with BDA in animals that had been pretreated with the serotonergic neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) to selectively destroy central serotonergic projections. Previous experiments demonstrated that after injection of BDA into the DRN of normal animals, labeled fibers descend to the vestibular nuclei within the mlf and in the ventricular fiber plexus. By contrast, in animals treated with 5,7-DHT, labeled fibers only descended to the vestibular nuclei within the ventricular plexus. In 5,7-DHT-pretreated animals, BDA-labeled terminals were notably absent in fields within lateral MVN and LVN that showed dense labeling in untreated rats. These findings indicate that 5,7-DHT produces a selective loss of BDA-labeled fibers descending within the mlf and terminating within lateral regions of the vestibular nuclei indicates that this projection may be primarily serotonergic in nature.

# 5.2 INTRODUCTION

It is well established, based on experiments combining retrograde tracing with 5-HT immunostaining, that the DRN contains nonserotonergic projection neurons (Beitz et al., 1986; Ma et al., 1991; Petrov et al., 1992, 1994; Van Bockstaele et al., 1993; Kirifides et al., 2001; Kim et al., 2004). Our retrograde tracing studies (Halberstadt and Balaban, 2003; Chapter 3.0) demonstrate that the vestibular nuclei are targeted by projections originating from nonserotonergic DRN cells. Nonserotonergic DRN projections are neurochemically heterogeneous, and may contain a number of transmitter substances, including glutamate (Schwarz and Schwarz, 1992; Kiss et al., 2002), dopamine (Pohle et al., 1984; Descarries et al., 1986; Yoshida et al., 1989; Stratford and Wirtshafter, 1990; Hasue and Shammah-Lagnado, 2002), GABA (Ford et al., 1995; Jankowski and Sesack, 2002), and nitric oxide (Simpson et al., 2003). Although it is likely that non-5-HT DRN projections influence processing in target regions (Weiss and Pellet, 1982; Hajés-Korcsok and Sharp, 2002), very little is known about the anatomical organization of this pathway. A few studies have used a combination of anterograde tracing and 5-HT immunofluorescent labeling to examine the organization of nonserotonergic DRN projections (Kosofsky and Molliver, 1987; Aznar et Unfortunately, there is substantial variation in the concentration of 5-HT present in al., 2004). serotonergic fibers, and it has not been conclusively established that the absence of 5-HT immunofluorescent labeling can be used to unequivocally identify fibers as being nonserotonergic (Nielsen et al., 2006). Therefore, it is important to develop other methodologies that can be used to reliably anterogradely trace nonserotonergic DRN projections. Selective serotonergic neurotoxins, including 5,7-dihydroxytryptamine (5,7-DHT), have been shown to disrupt axonal transport in serotonergic neurons (Moore and Halaris, 1975; Halaris et al., 1976; Azmitia and Segal, 1978; Jacobs et al., 1978; Moore et al., 1978; Satoh, 1979; Araneda et al., 1980a,b; Zhou and Azmitia, 1983; Callahan et al., 2001). Therefore, following 5,7-DHT-induced denervation of central serotonergic neurons, it should be possible to selectively trace the projections of nonserotonergic DRN neurons using BDA. We used

this approach to specifically examine the course and topography of nonserotonergic raphe-vestibular projections originating in DRN.

#### 5.3 MATERIALS AND METHODS

# 5.3.1 Animals

All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh, which certifies compliance with National Institutes of Health and United States Department of Agriculture standards for humane animal utilization. All efforts were made to minimize the number of animals used and their suffering. Pairs of animals were housed in suspended caging at 22 °C with a 12-h light/dark cycle and *ad libitum* access to food and water.

# 5.3.2 Surgical Procedures

Twelve adult male Long-Evans rats (250–300 g; Charles River Laboratories) were anesthetized using a mixture of ketamine (50 mg/kg, i.m.), xylazine (6 mg/kg, i.m.), and acepromazine (0.5 mg/kg, i.m.). They were then fixed in a stereotaxic apparatus using ear bars and a bite bar. The rats were pretreated with a combination of nomifensine and desipramine to prevent damage to dopaminergic and noradrenergic projections, respectively (Björklund et al., 1975; Baumgarten et al., 1982; Caillé et al., 2002). Thirty min after injection of nomifensine maleate (15 mg/kg, i.p.) and desipramine hydrochloride (15 mg/kg, i.p.), rats were administered 5,7-DHT creatinine sulfate (Fluka) by the intracerebroventricular (i.c.v.) route. The injection was stereotaxically guided, and was made through a burr hole in the cranium. Using a 20  $\mu$ l Hamilton syringe, 150  $\mu$ g 5,7-DHT (dose calculated as the free base: 1  $\mu$ g free base = 2.099  $\mu$ g 5,7-DHT creatinine sulfate) in 15  $\mu$ l 0.9% sterile saline containing 0.2% ascorbic acid was injected

into the left lateral ventricle over 15 min. This dosage of 5,7-DHT has previously been shown to produce extensive denervation of serotonergic projections (Björklund et al., 1975; Semple-Rowland et al., 1996; Wang et al., 2004). The burr hole was then closed with Gelfoam and the scalp incision sutured.

Seven days later, the animals were anesthetized using a mixture of ketamine (50 mg/kg, i.m.), xylazine (6 mg/kg, i.m.), and acepromazine (0.5 mg/kg, i.m.), and then fixed in a stereotaxic apparatus using ear bars and a bite bar. A burr hole was drilled in the cranium, and a solution of 7.5% BDA (10,000 MW; Molecular Probes, Eugene, OR, USA) in 10 mM phosphate-buffer containing 0.5 M NaCl, pH 7.0, was injected iontophoretically (4  $\mu$ A positive pulsed square wave, 15 s duty cycle, 15 min) into DRN using glass micropipettes (~40  $\mu$ m tip diameter). The micropipette was positioned at an angle 26° to the vertical plane, and aimed at the following stereotaxic coordinates: 1.0 mm posterior to the interaural line, 0 mm lateral, and 6.5 mm ventral to the skull surface, according to the atlas of Paxinos and Watson (1998). The burr hole was then closed with Gelfoam and the scalp incision sutured.

# 5.3.3 Euthanasia, Fixation and Sectioning

After a 5 day survival period, rats were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.), and perfused transcardially with PBS followed by PLP fixative. The brains were extracted from the skulls, post-fixed overnight at 4 °C, cryoprotected for 48–72 h at 4 °C in 30% sucrose–PBS, and 35 µm sections were cut in the transverse plane on a freezing–sliding microtome.

# 5.3.4 SERT Immunostaining

SERT-immunoreactivity was visualized using previously established procedures (Halberstadt and Balaban, 2003). After washing with distilled water ( $5 \times 5$  min), free-floating sections were treated with 1% dimethylsulfoxide in PBS for 90 min, rinsed with distilled water, and incubated for 10 min with 0.9% H<sub>2</sub>O<sub>2</sub> in PBS. The sections, after being washed with distilled water and then with PBS, were treated

overnight with blocking buffer (PBS with 2% BSA and 0.2% Triton X-100) containing 5% nonfat dry milk. Sections were then rinsed with PBS to remove the nonfat dry milk, treated with avidin/biotin block (Avidin/Biotin Blocking Kit, Vector Laboratories) according to the manufacture's instructions, rinsed with PBS, and incubated for 48 h at 4 °C in a mouse monoclonal anti-SERT antibody (1:1,000; Chemicon International, Temecula, CA, USA) diluted with blocking buffer. Following extensive washing with PBS, the sections were treated for 1 h with a biotinylated horse anti-mouse antibody (1:100; Vector Laboratories) diluted in PBS containing 2% BSA. After five washes with PBS, sections were incubated in ABC reagent (Vectastain ABC Elite Kit, Vector Laboratories) for 1 h, washed repeatedly with PBS, and transferred to 50 mM Tris buffer (pH 7.2) for 10 min. Immunoreactivity was then visualized by reaction with DAB chromagen (8.3  $\mu$ l 30% H<sub>2</sub>O<sub>2</sub> and 20 mg DAB in 100 ml 0.5 M sodium acetate buffer, pH 6.0). Sections were rinsed with PBS and mounted on gelatin/chrome alum-subbed slides. After air-drying overnight, the sections were dehydrated through a graded series of ethanol, cleared with xylene, and coverslipped with DPX Mountant (Fluka).

#### 5.3.5 BDA Staining

A one-step ABC procedure was used to visualize BDA labeling. Free-floating sections were washed with distilled water ( $3 \times 5$  min) and then treated with 0.9% H<sub>2</sub>O<sub>2</sub> in distilled water for 3 min. The sections were washed with PBS ( $4 \times 3$  min), and pre-incubated with 0.5% Triton X-100 in PBS for 1 h at room temperature. Following extensive washing with PBS ( $10 \times 3$  min), sections were incubated in ABC reagent (Vectastain ABC Elite Kit, Vector Laboratories) for 1 h at room temperature. After five washes with PBS, sections were rinsed with 50 mM Tris buffer (pH 7.2) for 10 min, and reacted with nickel-enhanced DAB chromagen (5 g ammonium nickel(II) sulfate, 40 mg DAB, and 16.6 µl 30% H<sub>2</sub>O<sub>2</sub> in 200

ml 0.5 M sodium acetate buffer, pH 6.0). Sections were rinsed with PBS and mounted on gelatin/chrome alum-subbed slides. After air-drying overnight, the sections were dehydrated through a graded series of ethanol, cleared with xylene, and coverslipped with DPX Mountant (Fluka).

#### 5.3.6 Analysis

Digital images of BDA injection sites, anterograde labeling, and SERT immunostaining were prepared using a Nikon Eclipse E600N microscope equipped with a Spot RT Monochrome camera (Model 2.1.1, Diagnostic Instruments, Inc., Sterling Heights, MI, USA). The images were captured on a Pentium-based computer running MetaMorph software (Ver. 6.1r4, Universal Imaging Corp., Downingstown, PA, USA). Adobe Photoshop 7.01 was used for brightness and contrast adjustments and cropping.

Camera lucida drawings were prepared using an Olympus BH-2 microscope ( $20 \times$  objective) equipped with a drawing tube. The full-size drawings were reduced by 50%, traced in India ink, and scanned at 600–800 dpi in grayscale mode.

## 5.4 **RESULTS**

# 5.4.1 Effect of 5,7-DHT on SERT Immunostaining of DRN Cells

The effect of administration of 5,7-DHT by the i.c.v. route on serotonergic neurons in the DRN was examined using SERT immunostaining in four of the 5,7-DHT-pretreated rats injected with BDA. Sections from normal animals (n = 4) and from 5,7-DHT-pretreated animals that had received an iontophoretic injection of BDA into the DRN (n = 4) were stained simultaneously for SERT-immunoreactivity. In control tissue from untreated animals, numerous SERT-positive cell bodies and fibers were found scattered throughout the DRN within the dorsomedial, ventromedial, and lateral

subdivisions (Figure 5.1A). However, 14 days after administration of 150 µg 5,7-DHT into the left lateral ventricle, there was a marked loss of SERT staining in the DRN (Figure 5.1B). In animals treated with 5,7-DHT, SERT-immunopositive neurons were completely absent within the DRN, and SERT-positive fibers were found in greatly reduced number. These findings indicate that i.c.v. administration of 150 µg 5,7-DHT has profound effects on SERT expression by serotonergic DRN cells.



Figure 5.1. Effect of intracerebroventricular 5,7-DHT on SERT immunostaining in the DRN.

(A) In normal animals, numerous SERT-positive cell bodies, dendrites, and fibers are localized throughout DRN.(B) Fourteen days after administration of 5,7-DHT there is a marked loss of SERT immunostaining in DRN. The medial longitudinal fasciculus (mlf) is also indicated. Scale bars=200 μm.



Figure 5.2. Photomicrographs of the BDA injection sites in the DRN in (A) case DHT-222C, (B) case DHT-324B, (C) case DHT-324A, (D) case DHT-214A, (E) case DHT-331B, and (F) case DHT-709A.

The medial longitudinal fasciculus (mlf) is also indicated. Scale bars=200  $\mu m.$ 

# 5.4.2 BDA Anterograde Tracing of Nonserotonergic DRN Projections

# 5.4.2.1 Injection sites

Of the twelve animals pretreated with 150 µg 5,7-DHT i.c.v. that received iontophoretic BDA injections, seven had injection sites that were centered in the DRN with minimal involvement of adjacent structures. The BDA injection sites are illustrated in Figure 5.2. The BDA injection sites displayed a dense central core of labeled neuronal somata and neuropil surrounded by a peripheral region containing scattered neuronal perikarya with dendrites that extend into the core. These sites were generally ovoid and relatively small, with the diameter of the central core typically measuring 200 µm in the mediolateral direction and extending for a similar distance rostrocaudally across transverse sections. Injection cases DHT-214A, DHT-222B, DHT-324B and DHT-331B involved both the caudal and intermediate levels of the DRN, and injection sites DHT-222C, DHT-324A, and DHT-709A were centered within the intermediate part of DRN. The BDA injection sites were centered within different subdivisions of the DRN, although they often spanned multiple subdivisions. Injection cases DHT-222C (Figure 5.2A) and DHT-324B (Figure 5.2B) were relatively confined within DRNI. Case DHT-324A (Figure 5.2C) was situated in DRNI with some spread into the mlf. The injection sites in cases DHT-214A (Figure 5.2D) and DHT-222B (not shown) were located in DRNI with some involvement of medial DRN subdivisions. The injection site in case DHT-331B (Figure 5.2E) was primarily located within DRNdm, but also involved DRNvm and bilateral aspects of DRNI. Finally, the tracer injection in case DHT-709A (Figure 5.2F) was made into DRNdm and the dorsal part of DRNvm.

Axons could be traced from the injection site to their terminations in the vestibular nuclei. The distribution of BDA-labeled fibers within five representative transverse sections spanning the rostrocaudal extent of the vestibular nuclei from cases DHT-222C, DHT-324B, DHT-324A, DHT-214A, DHT-331B and DHT-709A is illustrated in Figures 5.3–5.8. Transport ipsilateral to the injection site is illustrated to the right side of each figure. These camera lucida drawings reveal that the distribution of fibers and terminal regions was consistent across cases. Although there was little difference in the overall



# Figure 5.3. Distribution of anterogradely labeled fibers in a series of transverse sections spanning the rostrocaudal extent of the vestibular nuclei from case DHT-222C.

BDA-labeled fibers are charted in camera lucida drawings of five progressively caudal sections (A–E) located at the indicated AP level (relative to bregma). Transport ipsilateral to the injection site is illustrated on the right side of the figure. (Abbreviations: DTN, dorsal tegmental nucleus; g7, genu of facial nerve; IVN, inferior vestibular nucleus; LC, locus coeruleus; LVN, lateral vestibular nucleus; mlf, medial longitudinal fasciculus; MVN, medial vestibular nucleus; NTS, nucleus tractus solitarius; PBN, parabrachial nucleus; PrH, nucleus prepositus hypoglossi; scp, superior cerebellar peduncle; sg, supragenual nucleus; SLC, nucleus subcoeruleus; SUT, supratrigeminal nucleus; SVN, superior vestibular nucleus; y, group y).



Figure 5.4. Distribution of anterogradely labeled fibers in a series of transverse sections spanning the rostrocaudal extent of the vestibular nuclei from case DHT-324B. The format is identical to Figure 5.3.



Figure 5.5. Distribution of anterogradely labeled fibers in a series of transverse sections spanning the rostrocaudal extent of the vestibular nuclei from case DHT-324A. The format is identical to Figure 5.3.



Figure 5.6. Distribution of anterogradely labeled fibers in a series of transverse sections spanning the rostrocaudal extent of the vestibular nuclei from case DHT-214A. The format is identical to Figure 5.3.



Figure 5.7. Distribution of anterogradely labeled fibers in a series of transverse sections spanning the rostrocaudal extent of the vestibular nuclei from case DHT-331B. The format is identical to Figure 5.3.



Figure 5.8. Distribution of anterogradely labeled fibers in a series of transverse sections spanning the rostrocaudal extent of the vestibular nuclei from case DHT-709A. The format is identical to Figure 5.3.

pattern of labeling, the density of labeled fibers did vary between cases. However, there was no obvious relationship between the number (or density) of fibers anterogradely labeled in the vestibular nuclei and the mediolateral, dorsoventral or anteroposterior location of the injection site.

# 5.4.2.2 Course of BDA-labeled fibers descending from DRN

Fibers descended from DRN to the vestibular nuclei within the ventricular plexus, primarily on the side ipsilateral to the injection site. Within the caudal part of DRN, fine labeled fibers coursed dorsolaterally from DRN into the PAG, adjacent to the border of the cerebral aqueduct. As these fibers descend within the ventricular and periventricular plexus, some of them gradually fanned out dorsally along the edge of the fourth ventricle. Within the rostral pons—especially at AP levels immediately caudal to the part of DRN that was designated as group B6 by Dahlström and Fuxe (1964)—a number of fine fibers exited dorsolaterally from the ventricular fiber plexus and entered the caudal LC, where many of these fibers ramified heavily (Figure 5.9A). A group of the fibers traversed across caudal LC. When these fibers emerged from the lateral margin of LC they turned ventrolaterally before terminating in the rostral aspect of SVN (Figure 5.9B). This pattern of labeling was apparent in all injection cases, although it was especially pronounced in cases DHT-222C and DHT-324A (see Figure 5.3B and Figure 5.5B, respectively). Also within the rostral pons, an extensive fiber network was located on the ventral border of the fourth ventricle (see level A of Figures 5.3–5.8). At this AP level, a dense plexus of extremely convoluted, highly-branched, fine-caliber varicose fibers extended ventrally (and to a lesser extent ventrolaterally) from the ventricular plexus (Figure 5.9C). Large numbers of fibers then traversed the posterodorsal tegmental nucleus, mlf, and to a smaller extent, nucleus subcoeruleus. Further caudally (at levels B and C of Figures 5.3-5.8), this fiber network provided dense innervation to the supragenual nucleus and light innervation to the genu of the facial nucleus. This fiber network also innervated nucleus

prepositus hypoglossi (PrH), and this innervation was especially dense in cases DHT-324B (Figure 5.4E), DHT-324A (Figure 5.5E), and DHT-331B (Figure 5.7E).

As shown in Chapter 4.0, injection of BDA into the DRN of normal animals labeled numerous larger caliber nonvaricose fibers that descend to the vestibular nuclei within the mlf. Importantly, after treatment with 5,7-DHT, although labeled fibers could be seen traversing the mlf, no labeled fibers were observed descending from DRN within mlf (Figure 5.9C, D). This finding was consistent across all 5,7-DHT-pretreated cases, and the only labeled fibers that were detectable within mlf belonged to the network of fine fibers that extends ventrally from the ventricular plexus across the mlf.

#### 5.4.2.3 Morphological appearance of labeled fibers within the vestibular nuclei

After injection of BDA into the DRN of 5,7-DHT-pretreated animals, fine and very-fine caliber fibers were labeled anterogradely within the vestibular nuclei. Examples of labeled fibers in SVN and MVN are shown in Figure 5.10A, B, respectively. These small caliber labeled fibers had pleomorphic varicosities and were identical in appearance to the small caliber fibers labeled with BDA in untreated animals (see Chapter 4.0). Although larger caliber varicose and nonvaricose fibers were found to innervate the vestibular nuclei in normal animals, these types of labeled axons were rarely observed within the vestibular nuclei in animals treated with 5,7-DHT.

#### 5.4.2.4 Distribution of Labeled fibers within the vestibular nuclei

The terminal innervation of the vestibular nuclei was located primarily ipsilaterally to the injection site when the injection site was confined unilaterally. The projections were more symmetric in the injection cases that involved bilateral aspects of DRN (*i.e.*, DHT-331B and DHT-709A). Within the vestibular nuclei, the highest density of labeled fibers was found within the medial portions of MVN. The fibers in MVN were of fine caliber and appeared to originate from the ventricular plexus. In all injection cases, a plexus of labeled fibers was observed along the ventricular surface of MVN, and fibers from this ventricular plexus were found to extend laterally into periventricular regions of MVN (Figure 5.10C).



**Figure 5.9. Photomicrographs of anterogradely labeled fibers descending from DRN to the vestibular nuclei.** (A) Within the rostral pons, fine caliber fibers coursing dorsolaterally from the ventricular fiber plexus ramify within caudal LC. Note the absence of heavier caliber fibers within LC. (B) Fine caliber fibers from the ventricular plexus also innervate rostral SVN. (C) In the rostral pons, an extensive fiber network extends ventrally and ventrolaterally from the ventricular plexus, with large numbers of fibers traversing the posterodorsal tegmental nucleus (DTN) and the medial longitudinal fasciculus (mlf). Note the absence of heavier caliber fibers descending in the mlf pathway. (D) Further caudally, the density of this fiber network declines, and it provides only light innervation to the genu of the facial nucleus (g7). Scale bars=50 µm in A and B, 100 µm in C and D.



Figure 5.10. Photomicrographs of BDA-labeled fibers and terminals within the vestibular nuclei.

(A) Example of a fine caliber varicose fiber in SVN. (B) Example of a fine caliber varicose fiber in MVN. (C) Distribution of anterograde labeling in MVN. Labeled fibers extend laterally from the ventricular plexus (indicated by the asterisk), innervating the medial aspect of MVN. Note that much less anterograde labeling is present further laterally in MVN. Also note the complete absence of heavier caliber fiber labeling within MVN. Scale bars=20 µm in A and B, and 50 µm in C.

Compared with untreated animals, more labeled fibers were found in the periventricular and medial regions of MVN in 5,7-DHT-treated animals. This is clearly demonstrated by comparing Figure 4.09E with Figure 5.10C. Although anterograde labeling was detected throughout the rostrocaudal extent of medial MVN, labeled fibers were more numerous in rostral MVN (see level C of Figures 5.3–5.8) and mid-level MVN (see level D of Figures 5.3–5.8) compared with caudal MVN (see level E of Figures 5.3–5.8).

Scattered labeled axons were also observed in the dorsolateral and ventrolateral aspects of MVN in all cases. However, as illustrated in Figure 5.10C, many fewer axons were present in lateral regions of MVN than in medial MVN. This pattern of labeling is in clear contrast to that observed in normal animals (Chapter 4.0), where BDA-labeled fibers were especially numerous in lateral MVN (see Figure 4.09E).

Even though the labeled fibers were densest within the MVN of animals pretreated with 5,7-DHT, labeled fibers were also observed further laterally, particularly in rostral portions of the vestibular nuclei. The ventral aspect of rostral SVN received a dense innervation (Figures 5.9B and 5.10A); this projection was especially evident in case DHT-222C (see Figure 5.3B). It was also not unusual to find labeling within more caudal parts of SVN, although the innervation was typically sparser in caudal SVN compared with rostral SVN. However, in several injection cases (particularly cases DHT-222C and DHT-324B), relatively large numbers of labeled fibers ramified within caudal SVN, following a dorsolateral course from the ventricular plexus of dorsal MVN into SVN. The fiber density in both rostral LVN (level C of Figures 5.3–5.8) and caudal LVN (level D of Figures 5.3–5.8) was typically light, although cases DHT-214A, DHT-22C, and DHT-324B did show some labeling in LVN. The lowest density of fibers was present throughout IVN. Although in some injection cases a few labeled fibers were noted in IVN, in the majority of cases IVN was almost completely devoid of fibers.

#### 5.5 **DISCUSSION**

This study is the first to demonstrate that it is possible to trace the projections of nonserotonergic DRN neurons anterogradely (and selectively) by using BDA in combination with the serotonergic neurotoxin 5,7-DHT. The rationale for using such a combination is that pretreatment with 5,7-DHT eliminates the transport of BDA by serotonergic DRN projections while sparing transport of BDA by nonserotonergic DRN efferents. A substantial amount of experimental evidence demonstrates that selective 5-HT
neurotoxins prevent axonal transport in serotonergic cells but not in nonserotonergic cells. Moore and colleagues were the first investigators to test the hypothesis that it was possible to selectively trace the projections of nonserotonergic raphe neurons by pretreating the animals with a 5-HT neurotoxin (Moore and Halaris, 1975; Halaris et al., 1976; Moore et al., 1978). In experiments in which projections from the midbrain raphe nuclei (DRN and MRN) were anterogradely traced using [<sup>3</sup>H]proline, these investigators found that 5,6-DHT-pretreatment reduced the total radioactivity in target areas 73–95% and reduced axonal and terminal field labeling in a number of brain regions. Likewise, Azmitia and Segal (1978) observed that the transport of [<sup>3</sup>H]proline from DRN to forebrain areas innervated by DRN projections ascending in the medial forebrain bundle (MFB) is substantially reduced after microinjection of 5,7-DHT directly into the MFB. More recently, pretreatment with 5,7-DHT or the putative 5-HT neurotoxins fenfluramine and MDMA was shown to significantly decrease the anterograde transport of [<sup>3</sup>H]proline from the midbrain raphe nuclei (Callahan et al., 2001). It has also been shown—in rats treated with either 5,6-DHT or 5,7-DHT—that the retrograde transport of  $[^{3}H]$ 5-HT (Araneda et al., 1980*a*,*b*) and horseradish peroxidase (HRP) (Jacobs et al., 1978; Satoh, 1979; Zhou and Azmitia, 1983) is impaired in putative serotonergic fibers. Indeed, studies were able to demonstrate the presence of nonserotonergic neurons within the DRN by injection of HRP into the striatum (Jacobs et al., 1978) or forebrain (Baumgarten et al., 1974) of rats pretreated with 5,7-DHT. Importantly, electrophysiological findings indicate that putative nonserotonergic DRN cells are not damaged by 5,7-DHT (Aghajanian et al., 1978).

#### 5.5.1 Technical Considerations

#### 5.5.1.1 Efficacy of 5,7-DHT neurotoxic lesion

Bendotti et al. (1990) noted that cells in the ventromedial/intrafascicular DRN that are immunoreactive for tryptophan hydroxylase (TPH) and express TPH mRNA are spared after administration of 5,7-DHT in Sprague-Dawley rats. However, they noted that 5,7-DHT treatment produced a complete loss of serotonergic cells within DRNdm and DRNI. Similar findings have been reported using 5-HT

immunostaining (Hensler et al., 1994). Electrophysiological and fluorescence histochemical evidence also shows that putative serotonergic cells present in DRNvm are spared by 5,7-DHT (Aghajanian, 1978; Hajós and Sharp, 1996). Hence, it might be argued that at least a portion of the labeled fibers observed in the present study arise from 5,7-DHT-insensitive serotonergic DRNvm neurons rather than from nonserotonergic cells. However, the fact that we were unable to detect any SERT-immunoreactive DRN neurons in Long-Evans rats treated with i.c.v. 5,7-DHT strongly indicates that all serotonergic cells in the DRN are sensitive to 5,7-DHT. Indeed, our findings are consistent with those of Serrats et al (2005) who found that i.c.v. administration of 5,7-DHT in Wistar rats causes a complete loss of SERT mRNA expression throughout the DRN. Although investigators have presented biochemical and histochemical evidence that a minority of serotonergic fibers and terminals are spared by 5,7-DHT and other serotonergic neurotoxins (Stachowiak et al., 1986; O'Hearn et al., 1988; Brown and Molliver, 2000), it appears that these projections originate exclusively from MRN. Moreover, as fine serotonergic fibers are reported to be selectively vulnerable to the effects of serotonergic neurotoxins (O'Hearn et al., 1988; Brown and Molliver, 2000), it would be expected that any projections originating from a hypothetical population of 5,7-DHT-insensitive serotonergic DRN neurons would be of relatively heavier caliber. Therefore, the fact that after 5,7-DHT treatment there is a complete loss of the larger caliber DRN projections normally seen in untreated animals (see Chapter 4.0) provides additional support for the contention that the BDA labeling observed in this study is confined to nonserotonergic projections from the DRN. Rat strain differences in sensitivity to monoaminergic neurotoxins are not unprecedented (e.g., Schuerger and Balaban, 1995); our finding may be another example of this phenomenon.

The finding that animals treated with 5,7-DHT have a profound loss of SERT-immunoreactivity within DRN cells is consistent with earlier reports that after intracerebroventricular administration of 5,7-DHT there is a substantial reduction of both the level of SERT mRNA (Semple-Rowland et al., 1996) and the specific binding of the SERT ligand [<sup>3</sup>H]cyanoimipramine (Hensler et al., 1994) within the DRN. The observed loss of SERT protein expression by DRN neurons does not, by itself, demonstrate conclusively

that these cells have been destroyed. The mechanism of action of 5,7-DHT is believed to involve intraneuronal autoxidation to quinone derivatives that in turn catalyze the formation of cytotoxic reduced-oxygen species, resulting in cellular and metabolic damage (Tabatabaie et al., 1993). Despite the presence of 5,7-DHT-induced metabolic deficits which prevent the synthesis and/or expression of SERT protein, it is theoretically possible that these serotonergic neurons are otherwise intact. However, the presence of extensive terminal and neuronal degeneration within the DRN after intraventricular administration of 200 µg 5,7-DHT, as evidenced by Fink–Heimer silver-staining (Harvey, 1978), provides additional evidence that 5,7-DHT has a neurotoxic effect on the somata and projections of serotonergic DRN cells. Similarly, intraventricular 5,7-DHT has been shown to increase the expression of glial fibrillary acidic protein (O'Callaghan and Miller, 1993; Wang et al., 2004), a specific indicator of neuronal damage. Nevertheless, regardless of whether serotonergic DRN cells are actually destroyed by 5,7-DHT, the significant point remains that this agent selectively eliminated the anterograde transport of BDA within a specific subset of DRN projections (large caliber axons).

One important, and potentially counterintuitive finding of this investigation, is that often more fibers are labeled anterogradely with BDA in medial aspects of the vestibular nuclei in 5,7-DHT-pretreated animals compared with animals that were not treated with this neurotoxic agent. The observed increase in the density of labeling occurred despite the fact that 5,7-DHT treatment was found to *completely eliminate* the labeling of two classes of axons—medium caliber varicose fibers and large caliber non-varicose fibers—that are normally labeled after injection of BDA into the DRN. If it is assumed that degenerating serotonergic neurons no longer take up and accumulate BDA, one potential explanation for this phenomenon is that treatment with 5,7-DHT eliminates one group of DRN cells—serotonergic neurons—that would normally compete with nonserotonergic DRN cells for uptake of BDA. Therefore, compared with normal animals, it is possible that in animals treated with 5,7-DHT the soma and dendrites of nonserotonergic cells are exposed not only to higher concentrations of BDA, but are also exposed to BDA for longer periods of time. Furthermore, it is also possible that uptake of BDA by

nonserotonergic DRN neurons is facilitated by alterations in the density of neuropil and/or changes in cellular packing density that may occur as a byproduct of 5,7-DHT-induced degeneration of serotonergic neurons.

# 5.5.1.2 Comparison with other methodologies used to anterogradely trace nonserotonergic DRN projections

Other methodologies have also been used to visualize nonserotonergic projections from DRN. For example, a number of studies have combined BDA or PHA-L anterograde tracing with immunofluorescent staining for 5-HT in order to differentiate serotonergic and nonserotonergic DRN projections (Kosofsky and Molliver, 1987; Li et al., 2000; Thompson and Thompson, 2001; Aznar et al., However, we rejected this strategy for several reasons. First, the intensity of 5-HT-2004). immunostaining is dependent on the endogenous level of the transmitter (Datiche et al., 1995), and there is some evidence that changes in axonal concentrations of 5-HT can lead to a substantial underestimation of the density of 5-HT-positive fibers (Nielsen et al., 2006). Thus, when using this methodology, it may not be possible to differentiate between nonserotonergic fibers and serotonergic fibers that contain relatively low levels of 5-HT immunoreactivity. Further, because fluorophores tend to fade when illuminated, the use of fluorescence methods to detect BDA and 5-HT is not optimally suited for mapping projections in great detail, and the use of fluorophores also severely limits long-term analysis of labeling. Additionally, one advantage of BDA is that it can be detected using simple and reliable ABC procedures. Although detection of BDA and 5-HT using dual-fluorophores is by no means exceptionally difficult, this methodology is subject to confounding factors such as the possibility that the antibodies used to visualize 5-HT and BDA have not penetrated uniformly into the tissue. Therefore, there are several reasons why it is advantageous to develop anterograde tracing methods that are capable of selectively tracing nonserotonergic DRN projections.

#### 5.5.2 Innervation of the Vestibular Nuclei by Nonserotonergic DRN Projections

The results demonstrate that the vestibular nuclei receive a substantial innervation from nonserotonergic DRN neurons. This finding supports those of our retrograde tracing studies (Halberstadt & Balaban, 2003; Chapter 3.0), which revealed that between 28% and 44% of the DRN neurons labeled retrogradely from the vestibular nuclei do not contain 5-HT-immunoreactivity. It has been proposed that nonserotonergic DRN projections can influence the firing of neurons in the vestibular nuclei. One group has reported (Licata et al., 1995) that the ability of electrical stimulation of the DRN to evoke brief increases or deceases in the maintained activity of neurons in LVN and SVN is at best only weakly attenuated by microiontophoretic application of the 5-HT<sub>2A/2C</sub> antagonists methysergide and ketanserin. Based on this evidence, it was concluded that the methysergide- and ketanserin-insensitive responses to DRN stimulation are mediated by activation of nonserotonergic projections. Unfortunately, this conclusion is weakened by earlier findings published by these workers. Specifically, Licata et al. (1990, 1993a) had previously reported that inhibitory responses of LVN and SVN neurons to iontophoresis of 5-HT, the 5-HT<sub>1A</sub>/5-HT<sub>7</sub>-selective agonist 8-hydroxy-2-(N,N-dipropylamino)tetralin (8-OH-DPAT), or the nonselective 5-HT<sub>1</sub>/5-HT<sub>2</sub> agonist 5-methoxy-N,N-dimethyltryptamine (5-MeO-DMT) are not modified by the concurrent application of methysergide or ketanserin. Therefore, it is entirely possible that the short-duration electrophysiological responses to DRN stimulation observed by those workers are mediated by methysergide- or ketanserin-insensitive 5-HT receptors rather than by receptors for other transmitters. Nonetheless, because the neurochemical identity of the nonserotonergic DRN projections to the vestibular nuclei has not been elucidated, it is not clear what role input from nonserotonergic DRN cells would play in the regulation or modulation of activity in the vestibular nuclei.

As reported in Chapter 4.0, injection of BDA into the DRN of normal animals anterogradely labels (1) fine caliber fibers that descend to the vestibular nuclei within the ventricular and periventricular plexus, and (2) fibers of typically heavier caliber that descend to the vestibular nuclei within the mlf. Fine caliber axons that innervate the periventricular regions of MVN arise from the former pathway, whereas the latter pathway contributes heavier caliber axons to the rostrodorsal and caudoventral vestibular terminal fields. The present study reveals that after injection of BDA into the DRN of animals that were pretreated with 5,7-DHT, labeled fibers are observed descending to the vestibular nuclei within the ventricular plexus but not within the mlf. Thus, many fibers descending in the ventricular plexus DRN projection pathway are *spared* by 5,7-DHT treatment whilst fibers descending in the mlf DRN projection pathway are completely eliminated. Furthermore, after treatment with 5,7-DHT, heavier caliber labeled fibers were notably absent within the vestibular nuclei. The 5,7-DHT-induced loss of the descending mlf pathway, along with the heavier caliber fiber innervation that this pathway contributes to the vestibular nuclei, strongly indicates that these projections arise *exclusively* from serotonergic DRN cells. This conclusion is supported by the fact that large caliber 5-HT-immunopositive fibers are normally observed coursing between the mlf and the vestibular nuclei (see Figure 4.7A). Conversely, the fact that fine caliber DRN projections that descend within the ventricular plexus and terminate primarily within the periventricular regions of MVN persist after administration of 5,7-DHT argues that these fibers arise from nonserotonergic DRN cells. These findings indicate that nonserotonergic DRN projections descend to the vestibular nuclei within the ventricular plexus but not within the mlf pathway. Although not addressed specifically by this study, it cannot be excluded that fine caliber serotonergic projections may also descend to the vestibular nuclei within the ventricular plexus. Indeed, a dense plexus of SERTimmunoreactive fibers is present in the ventricular plexus of MVN (Halberstadt and Balaban, 2003; Figure 1.2).

It appears that there are significant differences in the distribution of serotonergic and nonserotonergic DRN projections within the vestibular nuclei. The anterograde labeling observed in this study—presumably nonserotonergic DRN projections—was generally confined to the periventricular regions of the vestibular nuclei, with only sparsely distributed labeling located further laterally. The exception to this was that in several cases there was moderate innervation of rostral SVN. Conversely, in animals that were not treated with 5,7-DHT, labeling was present in both medial and lateral regions of the vestibular nuclear complex (see Chapter 4.0). Therefore, it appears that the labeling in lateral portions of

the vestibular nuclei—except for rostral SVN—in normal animals is primarily serotonergic. This is consistent with the fact that the fibers descending in the mlf in normal animals—heavier caliber putative serotonergic fibers that are lost after treatment with 5,7-DHT—ramify primarily within regions of the vestibular nuclei that are not immediately adjacent to the border of the fourth ventricle. For example, in normal animals one group of BDA-labeled putative serotonergic fibers followed a course from the mlf laterally through LC before finally ramifying within SVN, a dorsolateral portion of rostral MVN, and the dorsal aspect of LVN. A second group of BDA-labeled putative serotonergic fibers exited the mlf at more caudal AP levels and coursed laterally into ventrolateral MVN, ramifying within the ventral aspect of caudal LVN and the ventrolateral parts of mid-level and caudal MVN. Furthermore, the absence of discrete rostrodorsal and caudoventral terminal fields in animals treated with 5,7-DHT indicates that the presence of serotonergic fibers is the major defining characteristic of these DRN terminal regions. In other words, although both serotonergic and nonserotonergic terminals are organized into two discrete terminal fields.

Our previous retrograde tracing study demonstrated that approximately equal numbers of 5-HTpositive and 5-HT-negative DRN neurons project to lateral portions of the vestibular nuclei (Halberstadt and Balaban, 2003). Although the present findings indicate that lateral aspects of the vestibular nuclei are much more heavily innervated by serotonergic DRN projections than by nonserotonergic projections, these two sets of findings are not necessarily incompatible. Although retrograde tracing can be used to determine approximately how many neurons in a given brain area project to the region where the tracer was injected, the number of neurons that are retrogradely labeled is not necessarily a measure of how densely the target region is innervated. Therefore, it is possible that even though equal numbers of serotonergic and nonserotonergic DRN neurons project to lateral aspects of the vestibular nuclei, the density of innervation by serotonergic fibers is greater because these projections are more highly branched compared with nonserotonergic projections. Two regions of the vestibular nuclei that are sources of ascending projections to PBN—SVN and the dorsal part of rostral MVN (Balaban, 1996; Porter and Balaban, 1997)—are targeted by nonserotonergic DRN projections. We have demonstrated that nonserotonergic DRN cells send collateralized projections to the vestibular nuclei and to CeA (see Chapter 3.0), two components of the vestibulo-PBN-forebrain pathway. The fact that nonserotonergic DRN projections target the region from which the vestibulo-PBN pathway originates supports our contention that the collateralized DRN projections to the vestibular nuclei and CeA are involved in regulating transmission through the vestibulo-PBN-forebrain network.

#### 5.5.3 Innervation of the Ventricular Plexus by Nonserotonergic DRN Projections

The fact that fibers within the ventricular plexus were labeled with BDA in animals pretreated with 5,7-DHT strongly indicates that these fibers are nonserotonergic. Previous retrograde tracing studies have provided evidence that nonserotonergic DRN cells project to the ventricular plexus (Larsen et al., 1996; Mikkelsen et al., 1997; Simpson et al., 1998). However, there is some controversy in the literature regarding the extent to which nonserotonergic DRN cells contribute projections to the ventricular plexus. Using retrograde tracing combined with immunofluorescence staining for 5-HT, Larsen and colleagues (Larsen et al., 1996) observed that almost all the DRN cells projecting to the lining of the third ventricle contain 5-HT. The same group later reported (Mikkelsen et al., 1997) that only approximately 50% of the cells in DRNdm and DRNvm that were retrogradely labeled after injection of CTb into the third ventricle are serotonergic, as revealed by 5-HT immunohistochemistry. Other workers have presented findings demonstrating that the vast majority of DRN cells projecting to the ependymal wall of the lateral ventricle are 5-HT-positive by immunofluorescence (Simpson et al., 1998). The authors of the latter report proposed that this discrepancy might be ascribed to differences in the sensitivity of the immunofluorescence and the immunohistochemical techniques that had been used to detect 5-HT. Nonetheless, the present anterograde tracing data provides additional support for the conclusion that at least a small but significant portion of the DRN projections to the ventricular plexus arise from neurons that do not contain 5-HT.

#### 5.5.4 Innervation of Locus Coeruleus by Nonserotonergic DRN Projections

As evidenced by the presence of a moderately dense plexus of labeled fibers, the LC is innervated by projections from nonserotonergic DRN cells. These nonserotonergic fibers are varicose and of fine caliber, and descend from the DRN to the LC within the ventricular fiber plexus. Interestingly, the same nonserotonergic fibers that innervate the LC also contribute projections to rostral SVN. These observations confirm earlier findings reported in the literature that indicated that the DRN–LC projection contains a nonserotonergic component. The finding that the ability of electrical stimulation of the DRN to increase extracellular levels of norepinephrine (NE) in the hippocampus is unaffected by treatment with 5,7-DHT led Hajés-Korcsok and Sharp (2002) to speculate that non-5-HT DRN projections exert an excitatory influence on the firing of noradrenergic cells in the LC. Indeed, retrograde tracing has revealed that approximately 50% of the DRN neurons projecting to the LC do not contain 5-HT-immunoreactivity (Kim et al., 2004).

As was noted in Section 4.5.3, the caudal LC is the source of a substantial coeruleo-vestibular noradrenergic projection that heavily innervates the dorsal and rostroventral parts of LVN, and moderately innervates SVN (Schuerger and Balaban, 1993, 1999). Although the present findings demonstrate that nonserotonergic DRN projections innervate LC, the density of nonserotonergic projections to the vestibular nucleus regions targeted by the coeruleo-vestibular pathway was weak (LVN) to moderate (SVN). Nonetheless, it appears that nonserotonergic DRN projections could exert a substantial modulatory influence on processing in those two vestibular regions via effects on the activity of vestibular-projecting LC cells.

#### 5.5.5 Functional Implications of Nonserotonergic DRN Projections to Vestibular Nuclei

Early studies combining retrograde tracing with 5-HT immunofluorescent staining provided the first indication that *both* serotonergic and nonserotonergic DRN neurons send projections to target areas (Steinbusch et al., 1981; Köhler and Steinbusch, 1982; Köhler et al., 1982). Numerous retrograde tracing studies have subsequently verified that this organizational feature holds true for many brain regions targeted by DRN projections (O'Hearn and Molliver, 1984; Beitz et al., 1986; Ma et al., 1991; Petrov et al., 1992, 1994; Van Bockstaele et al., 1993; Datiche et al., 1995; Kirifides et al., 2001; Halberstadt and Balaban, 2003; Kim et al., 2004). The present findings using BDA anterograde tracing support the contention that nonserotonergic DRN cells represent a class of DRN projection neurons. Although previous studies combining anterograde tracing and 5-HT immunofluorescent staining have demonstrated that some of the fibers anterogradely labeled from the DRN are 5-HT-immunonegative (Kosofsky and Molliver, 1987; Aznar et al., 2004), we believe that this study is the first to demonstrate that nonserotonergic DRN projections exist using a neurochemically-selective anterograde tracing methodology.

It is well known that in behaving cats, the activity of putative serotonergic DRN neurons increases across the sleep–wake–arousal cycle (Trulson and Jacobs, 1979; Sakai and Crochet, 2001) and reflects the level of ongoing motor activity (Trulson et al., 1981; Steinfels et al., 1983). Further, at least certain subpopulations of putative serotonergic DRN neurons are excited by the presentation of auditory and visual stimuli (Heym et al., 1982; Shima et al., 1986), and during rhythmic oral-buccal movements and in response to tactile stimulation (Fornal et al., 1996). However, although extensive electrophysiological work has been devoted to examining the firing properties of putative serotonergic DRN neurons in behaving animals (Trulson and Jacobs, 1979; Trulson et al., 1981; Heym et al., 1982; Steinfels et al., 1983; Fornal et al., 1996; Veasey et al., 1997; Guzman-Marin et al., 2000; Sakai and Crochet, 2001), much less effort has been made to characterize the response properties of nonserotonergic DRN neurons. Experiments in chloral hydrate-anesthetized rats have demonstrated that stimulation of the

sciatic nerve suppresses the firing of putative serotonergic DRN cells but increases the firing of putative nonserotonergic cells (Aghajanian et al., 1978). Recording from the DRN of behaving rats, Waterhouse and colleagues (Waterhouse et al., 2004) reported that although the firing activity of both serotonergic and nonserotonergic neurons was tied to the sleep-wake cycle, serotonergic DRN cells were generally insensitive to sensorimotor stimulation whereas nonserotonergic neurons displayed responses to specific sensory and motor events. These findings indicate that there are significant differences in the response properties of serotonergic and nonserotonergic DRN neurons. Given that serotonergic and nonserotonergic DRN projections are differentially distributed in the vestibular nuclei, the fact that serotonergic and nonserotonergic cells have different response properties may have profound implications regarding how processing in different regions of the vestibular nuclear complex is regulated by input from DRN. Indeed, it would appear that medial MVN and SVN are targeted by both serotonergic and nonserotonergic DRN projections, whereas lateral MVN and LVN are targeted primarily by serotonergic DRN projections. However, it will not be possible to determine how input from nonserotonergic DRN cells influences processing within the vestibular nuclei until it is known which transmitter substance(s) are synthesized by those nonserotonergic DRN projections.

### 6.0 SELECTIVE ANTEROGRADE TRACING OF SEROTONERGIC PROJECTIONS FROM THE DORSAL RAPHE NUCLEUS TO THE VESTIBULAR NUCLEI

#### 6.1 ABSTRACT

The vestibular nuclei are innervated by 5-HT- and SERT-immunopositive fibers, and retrograde tracing has demonstrated that these fibers originate primarily from DRN. The object of this investigation was to identify the terminal distribution of the serotonergic DRN-to-vestibular nuclei projections. The 5-HT neurotoxin 5,7-DHT (10 µg free base) was administered by intra-DRN microinjection to sixteen adult male Long-Evans rats in order to selectively lesion serotonergic projections from the DRN. After survival times of 18 h, degenerating fibers and terminals were visualized using amino–cupric–silver staining. In order to verify the placement of the 5,7-DHT injection site within the DRN, adjacent sections were immunostained for 5-HT and stained with Fluoro-Jade B, a fluorescent dye that selectively labels degenerating neurons. Silver-impregnated terminals and fibers were observed throughout the vestibular nuclei of 5,7-DHT-treated rats. Silver staining within the vestibular nuclei was dense along the border of the fourth ventricle within the vestibular place nuclei, large numbers of degenerating fibers and terminals were found in a region spanning laterally from the fourth ventricle and encompassing the rostral pole of SVN. Further caudally, relatively dense staining was observed in SVN, rostral LVN, and in the dorsal part of rostral MVN. Conversely, caudal regions of the vestibular nuclei, including caudal

LVN and caudal MVN were associated with lower densities of staining. Very few degenerating terminals were found in IVN. The overall topography of degenerating terminals was consistent with the distribution of SERT-positive axons within the vestibular nuclear complex.

#### 6.2 INTRODUCTION

It has been known since the beginning of the twentieth century that reduced silver methods can be used to stain normal and degenerating neuronal structures with high contrast (von Fajersztajn, 1901; Bielschowsky, 1904; Ramón y Cajal, 1904). Although it was soon demonstrated that silver staining could be used to trace axonal degeneration induced by an experimental lesion (Rasdolsky, 1923; Hoff, 1932), analysis of degeneration in silver stained sections was tedious because normal axons were also stained. This problem was eliminated after it was recognized that certain pretreatment steps would suppress the staining of normal neuronal elements, and "suppressive" silver stains were developed which selectively impregnate degenerating structures (Nauta and Gygax, 1954). A number of technical refinements were subsequently introduced, including the Fink-Heimer technique (Fink and Heimer, 1967) which permitted the visualization of degenerating terminals. Likewise, the cupric-silver method of de Olmos (1969) allowed degenerating terminals to be detected with even higher contrast and selectivity. These two silver staining techniques were widely used in the 1960s and 1970s to experimentally trace neuronal pathways, including the first demonstration of ocular dominance columns in visual cortex (Hubel and Wiesel, 1969). Furthermore, these methods have proven extremely useful in the detection of neurotoxin-induced damage (Hedreen and Chalmers, 1972; Maler et al., 1973; Harvey, 1978; Ricaurte et al., 1982, 1985; Balaban, 1985, 1992; Balaban et al., 1988: Molinari et al., 1996). The recently developed amino-cupric-silver staining procedure developed by de Olmos et al. (1994) has even greater sensitivity, contrast, reliability and reproducibility than previous methods, and this technique has been successfully used to visualize degeneration produced by neurotoxins such as acrylamide, glutamatergic excitotoxins, trimethyltin,

MDMA, MK-801, and ethanol (Jensen et al., 1993; de Olmos et al., 1994; Crews et al., 2000; Switzer, 2000; Bueno et al., 2003; LoPachin et al., 2003).

Cupric–silver staining methods have been successfully used to trace the axonal projections of degenerating nerve cells in great anatomical detail (Maler et al., 1973; Carlsen et al., 1982; Heimer et al., 1987; Balaban, 1988). One advantage of these methods is that degenerating processes are stained with remarkable morphological detail, and it has been demonstrated that cupric–silver degeneration staining allows for detection of even the finest processes and terminal arborizations (Balaban, 1992; de Olmos et al., 1994). Silver staining has been used to detect terminal and neuronal degeneration induced by the selective dopaminergic neurotoxin 6-hydroxydopamine (6-OH-DA) (Hedreen and Chalmers, 1972; Jeon et al., 1995), and Maler et al. (1973) were able to use silver staining to trace degeneration in the dopaminergic nigrostriatal pathway induced by microinjection of 6-OH-DA into the substantia nigra. These findings indicate that it may be possible, in animals treated with the selective serotonergic neurotoxin 5,7-DHT by intra-DRN administration, to selectively trace the projections of degenerating serotonergic DRN cells using silver staining. Importantly, neurotoxin-induced degeneration of DRN cells and terminals has been previously demonstrated using second generation suppressive silver staining techniques (Harvey, 1978; Balaban, 1985; Ricaurte et al., 1985), and workers have traced degenerating DRN and MRN projections using silver staining (Conrad et al., 1974; Massari et al., 1978).

The DRN is a source of extensive projections to the vestibular nuclei, and these projections originate from both serotonergic and nonserotonergic DRN neurons (Halberstadt and Balaban, 2003; Chapter 3.0). The results of our anterograde tracing experiments (Chapter 4.0; Chapter 5.0) indicate that there are substantial differences in the termination patterns of serotonergic and nonserotonergic DRN projections within the vestibular nuclei. BDA anterograde tracing demonstrated that DRN projections terminate within both periventricular and lateral regions of the vestibular nuclei (Chapter 4.0). By contrast, selective BDA tracing of nonserotonergic DRN projections revealed that this pathway primarily innervates the periventricular/medial aspect of the vestibular nuclei (Chapter 5.0). These findings indicate that 5-HT-containing fibers comprise a substantial portion of the DRN innervation present within

lateral aspects of the vestibular nuclei. However, immunostaining for 5-HT (Steinbusch, 1981, 1991; Harvey et al., 1993) and SERT (Halberstadt and Balaban, 2003; Figure 1.2) demonstrates that the medial aspects of the vestibular nuclei are densely innervated by serotonergic projections, and it is likely that these serotonergic projections arise primarily from DRN. Therefore, using the highly sensitive amino– cupric–silver staining technique, we have attempted to map the distribution of degenerating serotonergic terminals in the vestibular nuclei after direct microinjection of 5,7-DHT into the DRN.

#### 6.3 MATERIALS AND METHODS

#### 6.3.1 Animals

All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh, which certifies compliance with National Institutes of Health and United States Department of Agriculture standards for humane animal utilization. All efforts were made to minimize the number of animals used and their suffering. Pairs of animals were housed in suspended caging at 22 °C with a 12-h light/dark cycle and *ad libitum* access to food and water.

#### 6.3.2 Surgical Procedures

Serotonergic lesions restricted to individual raphe nuclei can be produced by intra-raphe microinjection of 5,7-DHT, and this methodology has been widely used to selectively lesion serotonergic projections from DRN, MRN, and RMg (Sawynok and Reid, 1988; Hensler et al., 1994; Compan et al., 1996; Gerard et al., 1996; Meyer-Bernstein and Morin, 1996; Harrison et al., 1997; Fletcher et al., 1999; Kaehler et al., 1999; Caillé et al., 2002).

Twenty-three adult male Long-Evans rats (250–300 g; Charles River Laboratories) were anesthetized using a mixture of ketamine (50 mg/kg, i.m.), xylazine (6 mg/kg, i.m.), and acepromazine (0.5 mg/kg, i.m.). The animals were then fixed in a stereotaxic apparatus using ear bars and a bite bar. The rats were pretreated with nomifensine and desipramine to prevent damage to dopaminergic and noradrenergic projections, respectively (Baumgarten et al., 1982; Caillé et al., 2002). A burr hole was drilled in the cranium, and 30 min after injection of nomifensine maleate (15 mg/kg, i.p.) and desipramine hydrochloride (15 mg/kg, i.p.), 10 µg of 5,7-DHT (dose calculated as the free base: 1 µg free base = 2.099 µg 5,7-DHT creatinine sulfate) in 0.5 µl of 0.9% sterile saline containing 0.2% ascorbic acid was injected into DRN using a 1 µl Hamilton syringe. The syringe needle was positioned at an angle 26° to the vertical plane, and aimed at the following stereotaxic coordinates: 1.0 mm posterior to the interaural line, 0 mm lateral, and 6.5 mm ventral to the skull surface, according to the atlas of Paxinos and Watson (1998). The burr hole was then closed with Gelfoam and the scalp incision sutured.

#### 6.3.3 Euthanasia and Fixation

After induction of an experimental lesion, the optimal postoperative survival for demonstrating neuronal degeneration varies depending on the part of the fiber system examined (de Olmos et al., 1981; Switzer, 2000). Detection of degeneration terminals is transitory and is maximal after short survival periods (the optimal period ranging from a few hours to a few days, depending on the specific fiber system), whereas longer survival periods are required to visualize degenerating axons and cell bodies. Based on reports that terminal degeneration induced by 5,7-DHT and other serotonergic neurotoxins is maximally detectable approximately 18 h after drug administration (Ricaurte et al., 1985; Commins et al., 1987; Jensen et al., 1993), we have chosen to use an 18 h post-injection survival time for these experiments. Rats were deeply anesthetized with sodium pentobarbital (100 mg/kg i.p.), perfused transcardially with rinse solution (0.4% sucrose, 0.8% NaCl, 0.4% dextrose and 0.023% CaCl<sub>2</sub> in 67 mM cacodylate buffer,

pH 7.2–7.4) followed by fixative (4% paraformaldehyde and 4% sucrose in 67 mM cacodylate buffer, pH 7.2–7.4). After a 24 h period of storage at 4 °C, the brains were extracted from the skulls and cryoprotected in perfusion fixative containing 30% sucrose at 4° C for 5–7 days.

#### 6.3.4 Preliminary Amino–Cupric–Silver Staining Experiments

Due to the fact that it has not been conclusively demonstrated that silver staining is capable of visualizing degenerating serotonergic terminals, the ability of the amino-cupric-silver staining technique to detect terminal degeneration induced by microinjection of 5,7-DHT into the DRN was first assessed in a preliminary series of animals (n = 5). These animals were injected with 5,7-DHT and perfused as described above (see Sections 6.3.2 and 6.3.3, respectively). After being extracted from the skulls and cryoprotected, the brains from these animals were embedded in a 12% gelatin-30% sucrose-67 mM cacodylate buffer mixture and fixed in perfusion fixative containing 30% sucrose for an additional 2-4 days at 4 °C. The brains were then sectioned in the coronal plane on a freezing-sliding microtome at 40 μm, and the sections stored in perfusion fixative at 4 °C for a minimum of 3 days before being silver stained using the amino-cupric-silver staining procedure developed by de Olmos et al. (1994). All procedures were performed at room temperature unless otherwise noted. Free-floating sections were prepared for silver staining by rinsing overnight in distilled water. Sections were then rinsed in fresh distilled water ( $3 \times 20$  min), and transferred to a compartmented staining dish containing 130 ml of warm (48 °C) pre-impregnation solution (0.2 M boric acid/0.05 M sodium tetraborate buffer (pH 8.5), containing 4.6 mg/ml silver nitrate, 15 µl/ml 0.5% cupric nitrate, 0.15 µl/ml 10% cadmium nitrate, 46 µl/ml pyridine, and 24.6 µg/ml allantoin); the sections were left in this solution for 45 min at 48 °C with continuous agitation. After the sections had cooled to 22–23 °C, they were agitated at this temperature for an additional 2 h. Sections were transferred through two changes of anhydrous acetone with constant agitation (~60 s total), and then incubated with freshly prepared diamine-silver solution (each 50 ml contains: 20 ml distilled water, 1.5 g silver nitrate, 14 ml 100% ethanol, 1.5 ml acetone, 11.5 ml 0.4%

lithium hydroxide, and 2.5 ml concentrated ammonium hydroxide) for 25 min. Thereafter, the sections were treated with freshly prepared reducing solution (1.1 ml 10% neutral buffered formalin, 0.65 ml 1% citric acid monohydrate, and 9 ml 100% ethanol, raised to a volume of 90 ml with distilled water, and warmed to 32 °C) for 25 min with continuous agitation, and rinsed with distilled water ( $4 \times 15$  min). After pre-bleaching for 5 min in a solution of 63% ethanol and 0.016% potassium iodide in distilled water, transferred to bleaching solution (distilled water containing 6% potassium ferricyanide, 2% potassium chlorate, and 0.5% acetic acid), and the bleaching reaction quenched by rinsing with distilled water ( $2 \times 5$  min). Subsequently, the sections were agitated for 1–2 min in a solution of 0.058% potassium permanganate and 0.16% sulfuric acid, quickly rinsed in distilled water, stabilized in 2% sodium thiosulfate for 1 min with agitation, transferred to Rapid Fixer solution (Kodak concentrated Rapid Fixer solution A + B, diluted 1:6 in distilled water) for 5 min, and finally rinsed in distilled water ( $2 \times 5$  min). After being mounted on gelatin/chrome alum-subbed slides and then air-dried overnight, the sections were dehydrated through a graded series of ethanol, cleared with xylene, and coverslipped with DPX Mountant.

Examination of amino-cupric-silver stained material from this initial series of animals revealed that all of the 5,7-DHT injections had been correctly targeted. Furthermore, there was pronounced evidence of degeneration in terminal regions (argyrophilic terminals) and in the DRN (neuronal and terminal argyrophilia). Detailed analysis of silver stained material from these animals was not performed.

#### 6.3.5 Amino-Cupric-Silver Staining and 5-HT Immunostaining

After verifying in a preliminary series of animals that amino-cupric-silver staining can be used to visualize 5,7-DHT-induced terminal degeneration after an 18 hour survival period (see Section 6.3.4 above), the brains from sixteen 5,7-DHT-injected animals were shipped to NeuroScience Associates (Knoxville, TN, USA) for amino-cupric-silver staining, Neutral Red counterstaining, and 5-HT

immunostaining. NeuroScience Associates is equipped for large-scale processing by the de Olmos method, and this company can rapidly, reliably, and cost-effectively perform the staining. Briefly, after being transferred to NeuroScience Associates, the 16 brains were embedded in a solid gelatin matrix (Switzer, 2000), sectioned (coronal plane) at 40 µm, and sets of every sixth section were collected. One set of sections was stained using the amino–cupric–silver technique (de Olmos et al., 1994) and then counterstained using Neutral Red. A second set of sections was immunostained for 5-HT (see Section 4.3.5 for details). After completion of the staining procedures, the sections were mounted on gelatin/chrome alum-subbed slides, air-dried, dehydrated through a graded series of ethanol, cleared with xylene, and coverslipped with DPX Mountant (Fluka). A third set of sections was shipped back to our laboratory for Fluoro-Jade B staining (see Section 6.3.6 below).

#### 6.3.6 Fluoro-Jade B Staining

Fluoro-Jade B is a fluorescent polyanionic derivative of fluorescein that specifically labels degenerating neurons (Schmued and Hopkins, 2000). To determine whether Fluoro-Jade B can be used to visualize degenerating serotonergic DRN cells, one set of every sixth section from the brains of 5,7-DHT-injected rats was obtained from NeuroScience Associates and stained with Fluoro-Jade B. The protocol used for Fluoro-Jade B staining was developed by Schmued and Hopkins (2000). Briefly, the gelatin matrix-embedded sections were mounted on gelatin/chrome alum-subbed slides using a solution of 12 ml 0.1 M sodium acetate buffer (pH 5.0), 188 ml distilled water, and 50 ml of 95% ethanol. After being dried in an oven at 40 °C, the slides were rinsed with 95% ethanol, treated with mixture of 180 ml 95% ethanol and 20 ml 37% formalin for 5 min, rinsed again with 95% ethanol, and air dried. The slides were treated with 80% ethanol containing 1% sodium hydroxide for 5 min, immersed in 70% ethanol for 2 min, and washed with distilled water for 2 min. The slides were then transferred to 0.06% potassium permanganate for 10 min with agitation, washed with distilled water for 2 min, incubated for 20 min in a solution of 0.0004% Fluoro-Jade B (freshly prepared by adding 4 ml of a 0.01% stock solution of Fluoro-Jade B (Chemicon)

to 96 ml of 0.1% acetic acid), and then rinsed with distilled water ( $3 \times 1$  min). The slides were thoroughly dried in an oven at 40 °C, cleared with xylene, and coverslipped with DPX Mountant. Sections stained with Fluoro-Jade B were examined under fluorescent illumination, using a Nikon Eclipse E600N microscope equipped with a DAPI/FITC/Texas Red triple-band filter cube (Chroma Technology Corp., Rockingham, VT, USA) and a 469–499 nm excitation filter (Chroma).

#### 6.3.7 Light Microscopic Examination of Amino–Cupric–Silver Staining

The amino–cupric–silver staining technique is more sensitive, specific, and reliable than earlier reduced silver staining methods (de Olmos et al., 1994). Silver-impregnated neuronal somata, dendrites, fibers, and terminals appear as a black silver precipitate against a light gray background. Degenerating axon terminals are typically apparent as an accumulation of small black argyrophilic particles (de Olmos et al., 1981). These terminal fragments may be aligned serially, and are sometimes interconnected. The fine particulate argyrophilia of nerve terminals typically appears within hours of the insult, followed by a longer-lasting degeneration of the parent axons.

#### 6.3.8 Verification That the 5,7-DHT Injection Site was Located Within the DRN

The presence of a 5,7-DHT injection site within the DRN was verified using three criteria: (1) regional loss of 5-HT-immunopositive cell bodies; (2) regional distribution of silver-impregnated neuronal perikarya, as demonstrated by the amino–cupric–silver staining technique; and (3) regional distribution of Fluoro-Jade B-positive neuronal perikarya. Cases in which the presence of a 5,7-DHT injection site could not be verified using 5-HT immunostaining *and* by at least one of the latter two methodologies were treated as control injections for purposes of analysis.

#### 6.3.9 Digital Image Capture

Digital images of silver staining and 5-HT immunostaining were prepared using a Nikon Eclipse E600N microscope equipped with a Spot RT Monochrome camera (Model 2.1.1, Diagnostic Instruments, Inc., Sterling Heights, MI, USA). The images were captured on a Pentium-based computer running MetaMorph software (Ver. 6.1r4, Universal Imaging Corp., Downingstown, PA, USA). Adobe Photoshop 7.01 was used for brightness and contrast adjustments and cropping.

#### 6.4 **RESULTS**

#### 6.4.1 Control 5,7-DHT Injection Cases

Of the sixteen 5,7-DHT-injected animals analyzed for this study, thirteen met the criteria described in Section 6.3.8 for verification that the 5,7-DHT injection site was located within the DRN. In each of the thirteen cases, the location of the 5,7-DHT injection site within the DRN was detectable using 5-HT immunostaining, silver staining, and Fluoro-Jade B staining. The three remaining cases, DHT-01, DHT-15, and DHT-16, served as control injection cases for purposes of comparison.

#### 6.4.2 5-HT Immunostaining

The effect of local microinjection of 5,7-DHT on the distribution of 5-HT-positive cell bodies in the DRN was examined using 5-HT immunostaining. In control animals, numerous 5-HT-immunopositive perikarya were scattered throughout the DRN within the dorsomedial, ventromedial, and lateral subdivisions (Figure 6.1A, B). After injection of 5,7-DHT directly into the DRN, the location of the injection site was clearly discernable as a region in which the intensity of somatodendritic 5-HT





Figure 6.1. 5-HT immunostaining in the DRN of control animals (A,B) and in animals in which 5,7-DHT was injected into the DRN (C–L).

A section from mid-level DRN (A,C,E,G,I,K) and from caudal DRN (B,D,F,H,J,L) is shown for each animal. (A, B) In control animals, numerous 5-HT-positive cell bodies are localized throughout DRN. (C, D) After injection of 5,7-DHT into the DRN in case DHT-02, the reduction in 5-HT immunostaining was heaviest in the dorsomedial and lateral subdivisions of mid-level DRN. (E, F) After injection of 5,7-DHT into the DRN in case DHT-04, the reduction in 5-HT immunostaining was heaviest in caudal DRN and the dorsomedial aspect of mid-level DRN. (G, H) 5-HT immunostaining in case DHT-06 was reduced most heavily in lateral DRN. (I, J) In case DHT-09, the reduction in 5-HT immunostaining was heaviest in a section of ventromedial DRN. (K, L) In case DHT-11, 5,7-DHT strongly reduced 5-HT immunostaining in both mid-level DRN (K) and caudal DRN (L). Scale bars=200 µm.



## Figure 6.2. 5-HT immunostaining in MVN from a control animal (A) and in MVN from an animal in which 5,7-DHT was injected into the DRN (B).

(A) In control animals, regions of the vestibular nuclei are innervated by a mixture of heavier caliber 5-HT positive fibers (indicated by arrows) and fine caliber 5-HT positive fibers. (B) There are very few 5-HT-immunopositive fibers in the vestibular nuclei after injection of 5,7-DHT into the DRN. Note that these sections were immunostained for 5-HT simultaneously. Scale bars=50  $\mu$ m.

immunostaining was markedly reduced or even completely eliminated (Figure 6.1C–L). In the majority of 5,7-DHT injection cases, there was a substantial loss of 5-HT-immunoreactivity throughout the dorsomedial and ventromedial subdivisions of caudal DRN (Figure 6.1F, L); however, in other cases, 5-HT immunostaining of cell bodies within caudal DRN was less extensively altered by 5,7-DHT (Figure 6.1D, H, J). Further rostrally, it was common to find that the staining intensity of neurons in DRNvm, DRNdm, and DRNI was not uniformly altered. For example, in cases DHT-02 (Figure 6.1C) and DHT-11 (Figure 6.1K), the apparent reduction of cell body 5-HT immunostaining was much greater in DRNdm and DRNI compared with DRNvm. Conversely, in case DHT-09, the loss of neuronal 5-HT immunostaining was most prominent in aspects of DRNvm, including the intrafascicular region (Figure 6.1E), while in case DHT-04, the loss of 5-HT immunostaining was greatest in DRNdm (Figure 6.1E),

In control animals, a mixture of fine caliber and heavier caliber 5-HT-positive fibers was observed within the vestibular nuclei (Figure 6.2A). This labeling was present throughout the vestibular nuclei, although it was much weaker in IVN compared with MVN, SVN and LVN. Importantly, microinjection of 5,7-DHT into the DRN resulted in a profound loss of 5-HT-immunoreactive fibers within terminal regions (Figure 6.2B). The extent to which 5-HT immunostaining within the vestibular nuclei was eliminated varied; in certain cases, 5-HT-immunoreactive fibers were almost completely absent (Figure 6.2B), whilst in other cases a few 5-HT-positive fibers were found scattered within the vestibular nuclei. These 5-HT-immunopositive fibers were typically of heavier caliber.

#### 6.4.3 Fluoro-Jade B Staining

Eighteen hours after local microinjection of 5,7-DHT, Fluoro-Jade B-positive neurons were detected in the DRN (Figure 6.3A). The neurons labeled with Fluoro-Jade B exhibited a bright yellow-green fluorescence under epifluorescence illumination. In control animals, no DRN neurons were stained with Fluoro-Jade B. The distribution of Fluoro-Jade B-positive neurons within the DRN (Figure 6.3A) closely matched that of argyrophilic neurons in adjacent sections that had been stained using the amino–cupric– silver technique (Figure 6.3C, D, E). Furthermore, the morphologic appearance of Fluoro-Jade B-positive neurons and argyrophilic neurons was similar to that of 5-HT-positive neurons from the DRN of normal animals (Figure 6.3B), and the Fluoro-Jade B-positive neurons and argyrophilic neurons were located within regions of DRN where serotonergic cells are known to be heavily clustered. Fluoro-Jade B-labeled fibers or terminals were not observed, either within the DRN or in regions known to be targeted by DRN projections, including the vestibular nuclei.

#### 6.4.4 Amino–Cupric–Silver Staining

#### 6.4.4.1 Somatodendritic degeneration

Argyrophilic neurons, as well as a substantial amount of dendritic and terminal debris, were apparent in the DRN after local microinjection of 5,7-DHT. The appearance of these degenerating DRN cells varied; typically, degenerating neurons were partially or completely filled with very fine silver granules (Figure 6.4A, B). Occasionally, however, neurons were densely impregnated and had a Golgi-like appearance (Figure 6.3C, D, E). There was also substantial case-by-case variation in the number and distribution of degenerating DRN neurons. In some injection cases, small numbers of degenerating neurons were found localized within an individual DRN subdivision. For example, neuronal degeneration was primarily confined to DRNvm in case DHT-02, and in case DHT-14 degenerating neurons were located in DRNdm. However, in the majority of injection cases, relatively large numbers of argyrophilic neurons were distributed over a substantial mediolateral, dorsoventral and rostrocaudal region of the DRN. Thus, in cases DHT-04, DHT-05, DHT-06, DHT-08, DHT-09 and DHT-12, cellular degeneration was apparent in the dorsomedial, ventromedial, and lateral DRN subdivisions. A similar pattern of labeling was especially prominent in the intrafascicular part of DRNvm.



Figure 6.3. After injection of 5,7-DHT into the DRN, degenerating neurons are located in regions of DRN where 5-HT immunopositive neurons are normally heavily clustered.

(A) Fluoro-Jade B staining in the DRN of 5,7-DHT injection case DHT-04. Several Fluoro-Jade B-stained neurons are located within the dorsomedial DRN subdivision, especially the ventral half of DRNdm. (B) Distribution of 5-HT immunopositive neurons within DRNdm of a control animal. Within the ventral aspect of DRNdm, 5-HT positive cells are heavily clustered within two lateral groups (indicated by arrows). (C) Amino-cupric-silver staining in DRNdm from case DHT-04; this section was located adjacent to the section in panel A. There are two clusters of densely silver-impregnated cells in ventral DRNdm. Note that the distribution of these argyrophilic neurons are located within the same region of ventral DRNdm where 5-HT positive neurons are heavily clustered (see panel B). The boxes in panel C indicate the areas shown enlarged in panels D and E. (D, E) High-power photomicrographs showing the morphology of argyrophilic somata in the same DRN section as panel C. Scale bars=100 µm in A, B and C, and 50 µm in D and E.



Figure 6.4. Photomicrographs of amino-cupric-silver stained sections, illustrating argyrophilic somatic and dendritic debris in DRN 18 hours after local microinjection of 5,7-DHT.

(A, B) Several DRN cells display silver-impregnation indicative of early degeneration. Scale bars=50 µm.

Serotonergic neurons in MRN are known to be susceptible to the effects of 5,7-DHT (Compan et al., 1996; Harrison et al., 1997). However, it was rare to find argyrophilic neurons in MRN, indicating that only small amounts of 5,7-DHT had diffused out of the DRN. Nonetheless, 5,7-DHT-induced degeneration of MRN neurons is unlikely to confound the interpretation of this study because the MRN does not send projections to the vestibular nuclei (Vertes et al., 1999; Halberstadt and Balaban, 2003).

#### 6.4.4.2 Terminal degeneration: locus coeruleus

Degenerating axonal debris were present in the caudal aspect of LC after administration of 5,7-DHT directly into the DRN (Figure 6.5A, B). These degenerating fibers often entered the LC from the adjacent ventricular plexus (Figure 6.5A); this finding indicates that some serotonergic DRN projections descend to the LC within the ventricular plexus. It is also noteworthy that the appearance of these silver-impregnated axons was very similar to that of the terminal branches of 5-HT-immunopositive axons (Figure 6.5C) and SERT-immunopositive axons (Figure 6.5D) that innervate the LC in normal animals.

#### 6.4.4.3 Terminal degeneration: vestibular nuclei

There was extensive axonal and terminal degeneration within the vestibular nuclei in animals treated with 5,7-DHT by intra-DRN injection. The degeneration was found in the rostral (Figure 6.6) and caudal (Figure 6.7) aspects of the vestibular nuclei, especially in regions corresponding to the location of the rostrodorsal and caudoventral terminal fields revealed using BDA tracing (see Chapter 4.0).

In the rostral half of the vestibular nuclei, silver-impregnated terminals were detectable in SVN (Figure 6.6A), LVN (Figure 6.6B), and MVN (Figure 6.6C, D). Extensive terminal degeneration was localized in SVN, and these terminals were typically of fine caliber (Figure 6.6A). Degenerating terminals were typically found to be only sparsely distributed in rostral LVN, and these terminals were of heavier caliber (Figure 6.6B). A substantial plexus of fine caliber degenerating terminals was located in the ventricular plexus, including the part of the ventricular plexus that borders rostral MVN (Figure 6.6C). In the medial aspect of rostral MVN, degenerating terminals were found extending laterally from the ventricular plexus, innervating the medial aspect of MVN. Further laterally in rostral MVN, there was a dense plexus of degeneration, involving both fine caliber and heavier caliber terminals (Figure 6.6D).

In the caudal half of the vestibular nuclei, silver-impregnated terminals were detectable in caudal LVN (Figure 6.7A) and caudal MVN (Figure 6.7B, C, D). As was seen in the rostral part of LVN, relatively sparse degeneration was present in caudal LVN (Figure 6.7A); however, although only heavier caliber degenerating terminals were located in rostral LVN, there was a mixture of both fine and heavier





(A, B) Photomicrographs of amino-cupric-silver stained sections illustrating degenerating axonal debris in caudal LC, 18 h after injection of 5,7-DHT into DRN. Several fine varicose axons are present (indicated by arrows); the axonal debris has a granular appearance, similar to that of the 5-HT- and SERT-immunopositive fibers shown in panels C and D. Note the presence of degenerating fibers in panel A that enter LC from the adjacent ventricular fiber plexus (denoted by an asterisk). (C) Photomicrograph of 5-HT-immunoreactive terminal branches of axons within LC and the adjacent ventricular fiber plexus (denoted by an asterisk) in a horizontal section (40  $\mu$ m) from a normal Long-Evans rat. (D) Photomicrograph of SERT-immunoreactive terminal branches of axons within LC and the adjacent ventricular fiber plexus (denoted by an asterisk) in a horizontal section (40  $\mu$ m) from a normal Long-Evans rat. 5-HT and SERT immunostaining was performed using established procedures (see Halberstadt and Balaban, 2003). Scale bars=50  $\mu$ m.



Figure 6.6. Photomicrographs of amino-cupric-silver stained sections illustrating axonal debris in the rostrodorsal vestibular terminal region, 18 hours after injection of 5,7-DHT into DRN.

(A) Fine caliber axon terminal debris in rostral SVN. (B) Degenerating heavier caliber axons and axonal debris in rostrodorsal LVN. (C) Fine caliber terminal degeneration in the ventricular plexus and the adjacent periventricular region of rostrodorsal MVN. (D) The lateral aspect of rostrodorsal MVN contains a mixture of fine and heavier caliber degenerating axons and axonal debris. (E) Drawings of the rostral half of the vestibular nuclei illustrating the locations of panels A, B, C and D within the rostrodorsal terminal field, which is shaded in grey. Note that the four photomicrographs are from sections at or near the indicated AP level (relative to bregma), and are from different injection cases. Scale bars=50 μm in A and C, and 100 μm in B and D.



Figure 6.7. Photomicrographs of amino-cupric-silver stained sections illustrating axonal debris in the caudoventral vestibular terminal region, 18 hours after injection of 5,7-DHT into DRN.

(A) Fine and heavier caliber degeneration in caudal LVN. (B) Fine caliber terminal degeneration in the ventricular plexus and the adjacent periventricular region of ventral MVN. Note the presence of terminals coursing laterally from the ventricular plexus into medial MVN. (C) The lateral aspect of ventral MVN contains a mixture of fine and heavier caliber degenerating axons and axonal debris. (D) Fine and heavier caliber debris in lateral caudal MVN. (E) Drawings of the caudal half of the vestibular nuclei illustrating the locations of panels A, B, C and D within the caudoventral terminal field, which is shaded in grey. Note that the four photomicrographs are from sections at or near the indicated AP level (relative to bregma), and are from different injection cases. Scale bars=50 μm in A and B, and 100 μm in C and D.

caliber degeneration in caudal LVN. Degenerating, fine caliber terminals were found in the ventricular plexus adjacent to caudal MVN (Figure 6.7B), but the density of these fibers was not as high as was found at more rostral levels of the vestibular nuclei; importantly, terminals in the ventricular plexus were found to course laterally into the medial aspect of caudal MVN (Figure 6.7B). The lateral aspect of caudal MVN contained a mixture of fine caliber and heavier caliber terminal degeneration (Figure 6.7C, D). Within both the rostral and caudal aspects of IVN, there was little or no terminal degeneration (data not shown).

#### 6.5 DISCUSSION

#### 6.5.1 Technical Considerations

#### 6.5.1.1 Detection of degenerating serotonergic terminals using silver staining

There has been some controversy in the literature regarding whether silver staining can be used to detect degenerating serotonergic terminals. The presence of degenerating fibers, but not terminals, has been demonstrated after electrolytic lesions of MRN and DRN using second generation suppressive silver staining techniques (Conrad et al., 1974; Massari et al., 1978). Although the fiber distribution was similar to that revealed by anterograde tracing with [<sup>3</sup>H]proline, it was not possible to determine the neurochemical identity of the degenerating fibers. Likewise, even though the Fink–Heimer silver staining method (Fink and Heimer, 1967) clearly demonstrates that argyrophilic cells are present in SLN after administration of the putative serotonergic neurotoxin PCA (at dosages that chronically deplete 5-HT from axon terminals), degenerating axons and terminals were not observed (Harvey et al., 1975, 1977; Harvey, 1978; Massari et al., 1978). Conversely, other workers observed degenerating fibers and terminals in striatum, hippocampus, thalamus, and neocortex after administration of PCA (Commins et al., 1987*a*), MDA (Ricaurte et al., 1985), and MDMA (Commins et al., 1987*b*; Scallet et al., 1988; Jensen

et al., 1993). Unfortunately, PCA and MDMA also produce neuronal argyrophilia in somatosensory cortex (Commins et al., 1987*a*,*b*; Jensen et al., 1993); since 5-HT-immunopositive neurons are not found in neocortex (Steinbusch, 1981), it appears that these agents are capable of damaging nonserotonergic cells. Indeed, Schmued (2003) has presented evidence that MDMA-induced degeneration of neocortical neurons is actually a consequence of drug-induced hyperthermia. Nonetheless, although the evidence demonstrating that silver staining can be used to visualize MDMA- and PCA-induced degeneration of serotonergic processes is equivocal, the fact that serious questions have been raised regarding whether PCA and MDMA are actually toxic toward serotonergic terminals (Rothman et al., 2003; Wang et al., 2005) argues that these psychostimulants should not be used to evaluate the detectability of serotonergic terminal degeneration.

Importantly, it has been reported that terminal degeneration induced by 5,7-DHT (and by the related serotonergic neurotoxin 5,6-DHT) can be visualized using the Fink–Heimer method. For example, three days after intraventricular administration of 5,7-DHT (200 µg), approximately 50% of the neurons in DRN, MRN and SLN were argyrophilic, and there was substantial terminal degeneration present within those three midbrain nuclei (Harvey, 1978). Likewise, Ricaurte et al. (1985) found degenerating terminals in hippocampus and striatum 18 h after intraventricular administration of 75 µg of 5,7-DHT. Robust staining of degenerating fibers and terminals, induced by 5,6-DHT, has been observed in the amphibian spinal cord (Mensah et al., 1974). Finally, it was noted by Moore (1981*b*) that degenerating fibers and terminals can be detected after intraventricular administration of 5,6-DHT, and the distribution of the degenerating serotonergic terminals are detectable using amino–cupric–silver staining, and furthermore demonstrate that silver staining can be used to trace the pattern of serotonergic terminal degeneration that results from injection of 5,7-DHT directly into the DRN.

The fact that argyrophilic neurons were present in the DRN of animals treated with 5,7-DHT confirms that this agent is toxic to neuronal perikarya in this raphe nucleus. Although it was not possible to verify the neurochemical identity of the degenerating DRN cells, it is highly likely that they are

serotonergic. First, in the presence of the catecholamine uptake blockers nomifensine and desipramine, 5,7-DHT has been shown to be selectively toxic toward serotonergic cells (Björklund et al., 1975; Baumgarten et al., 1981, 1982). Second, as shown in Figure 6.1, there was a marked loss of 5-HT-immunoreactivity in cell bodies in the DRN after injection of 5,7-DHT. Third, the morphology and intra-DRN distribution of Fluoro-Jade B-positive neurons and argyrophilic neurons was similar to that of 5-HT immunopositive cells (see Figure 6.3). These three factors argue that the degenerating DRN cells revealed using silver staining are serotonergic.

#### 6.5.1.2 Use of Fluoro-Jade B to detect degeneration induced by 5,7-DHT

Fluoro-Jade is a novel fluorescent marker of neuronal degeneration (Schmued et al., 1997); reportedly the pattern of labeling seen with Fluoro-Jade is virtually identical to the pattern of silver staining observed with the amino-cupric-silver method. Fluoro-Jade can be used to demonstrate neuronal degeneration induced by a variety of neurotoxins, including kainic acid, domoic acid, MK-801, ibogaine, and MPTP (Schmued et al., 1997; Freyaldenhoven et al., 1997). Fluoro-Jade B is a derivative of Fluoro-Jade that labels degenerating neurons with even higher sensitivity (Schmued and Hopkins, 2000; Benkovic et al., 2004). This study demonstrates that after treatment with 5,7-DHT, staining with Fluoro-Jade B can be used to visualize degenerating DRN neurons. Importantly, the distribution of DRN neuronal degeneration revealed by amino-cupric-silver staining and Fluoro-Jade B staining was very similar. Although it has been reported that Fluoro-Jade B labels degenerating terminals with high sensitivity, in our hands Fluoro-Jade B failed to demonstrate the presence of 5,7-DHT-induced terminal degeneration. By contrast, amino-cupric-silver staining clearly demonstrated that there was extensive terminal degeneration after administration of 5,7-DHT. It is possible that the ability of Fluoro-Jade B to detect degenerating serotonergic terminals is dependent on the post-lesion interval, and therefore the short survival period used in this study may not have been optimal for labeling degenerating serotonergic terminals with Fluoro-Jade B. Indeed, studies that observed labeling of degenerating terminals by Fluoro-Jade and Fluoro-Jade B have employed longer survival times (Schmued et al., 1997; Schmued and Hopkins, 2000).

Nonetheless, this finding indicates that toxicological and histochemical studies in which Fluoro-Jade B staining is the only methodology used to detect terminal degeneration may be susceptible to false-negative findings, especially if short survival periods are employed.

#### 6.5.1.3 Optimal post 5,7-DHT survival period

It is well established that terminal degeneration is relatively transient and develops much more quickly than does degeneration of non-terminal fibers or cell bodies (reviewed by de Olmos et al., 1981; Switzer, 2000); as such, short survival times are often required to optimally impregnate degenerating terminals by silver techniques, whereas longer survival times are required to detect degeneration of parent axons and cell bodies. Since the object of this study was to map the termination patterns of serotonergic DRN projections to the vestibular nuclei, we chose a relatively short post-5,7-DHT survival period (18 h) that had previously been shown to be compatible with the detection of 5,7-DHT-induced terminal degeneration using cupric-silver staining (Ricaurte et al., 1985). Unfortunately, the main drawback to using such a short survival period is that it is unlikely to permit the demonstration of degenerating axons of passage or of degenerating DRN neurons. Indeed, extensive terminal degeneration was present within regions targeted by the DRN-including the vestibular nuclei-but axons coursing between the DRN and its target regions were not impregnated. Therefore, an 18 h survival period can be used to map the distribution of degenerating serotonergic terminals but is not capable of tracing the course of DRN projection pathways. It is possible that the use of a longer survival period would permit the detection of both degenerating terminals and degenerating axons. Despite the short survival period used, silver staining revealed the presence of degenerative changes in DRN cells, typically evidenced by fine granular silver deposits within neuronal soma and dendrites. The increase in neuronal argyrophilia is potentially indicative of early degenerative processes, indicating that neurotoxic effects occur in DRN cells soon after exposure to 5,7-DHT.
# 6.5.1.4 Effect of 5,7-DHT on the expression of 5-HT immunoreactivity by serotonergic DRN neurons

Injection of 10 µg 5,7-DHT into the DRN substantially altered the intensity of 5-HT-immunoreactivity within that nucleus, and it was possible to estimate the location of the 5,7-DHT injection site based on the reduction or elimination of 5-HT immunostaining within DRN cell bodies. Previous studies have reported similar findings. One group of workers (Compan et al., 1996) estimated that the number of 5-HT-immunopositive DRN cells is reduced by 94% after local microinjection of 6.6 µg of 5,7-DHT. Another study, conducted in hamsters, reported that the number of 5-HT-immunoreactive cells in DRN was reduced 69% after injection of 14.4 µg 5,7-DHT into DRN (Meyer-Bernstein and Morin, 1996). Local application of 5.7-DHT within the DRN has also been shown to produce alterations in markers related to SERT. After microinjection of 8 µg of 5,7-DHT into the DRN, the binding of <sup>3</sup>H]cyanoimipramine to SERT was reduced by 80% (Hensler et al., 1994). Similarly, local injection of 8 µg of 5,7-DHT reduced SERT mRNA levels in the DRN by 90% (Gerard et al., 1996). However, although those four earlier studies employed long survival times (20 days, 7–10 days, 14 days, and 21 days, respectively), we used a much shorter survival period in our experiments (18 h); thus, it has now been shown that the cell bodies of serotonergic DRN cells display evidence of 5-HT depletion within hours of exposure to locally injected 5,7-DHT.

As was noted earlier (see Section 5.5.1.1), there is some controversy regarding whether neurons in the ventromedial DRN are susceptible to the neurotoxic effects of 5,7-DHT. However, after injection of 5,7-DHT directly into the DRN, there was a loss of 5-HT-immunoreactive somata within regions of DRN, including DRNvm. The ability of locally-applied 5,7-DHT to reduce 5-HT immunostaining of DRNvm cells confirms our previous observation (see Section 5.4.1) that SERT-positive (*i.e.*, serotonergic) DRNvm cells are sensitive to 5,7-DHT. Furthermore, the presence of argyrophilic neurons in DRNvm, and the fact that DRNvm neurons were labeled with Fluoro-Jade B, indicates that 5,7-DHT has a neurotoxic effect on serotonergic neurons located within this region of DRN.

### 6.5.2 Innervation of the Ventricular Plexus by Serotonergic Projections from DRN

It is well known that the ventricular ependyma are densely innervated by a plexus of serotonergic fibers. The presence of an indoleamine in fibers within the supraependymal plexus of rats was first demonstrated using the Falck-Hillarp histofluorescence technique (Lorez and Richards, 1973; Richards et al., 1973). Further studies revealed that the formaldehyde-induced yellow fluorescence within the supraependymal plexus was eliminated by lesions of the midbrain raphe nuclei (Aghajanian and Gallager, 1975) and by i.c.v. administration of 5,6-DHT (Lorez and Richards, 1976). Workers also presented autoradiographic findings showing that fibers in the sub- and supraependymal plexus selectively accumulate [<sup>3</sup>H]5-HT when it is administered by continuous intraventricular perfusion in the presence of a monoamine oxidase inhibitor (Chan-Palay, 1976). Soon thereafter, 5-HT immunostaining confirmed that the supraependymal plexus contains 5-HT-immunopositive fibers (Steinbusch, 1981).

It is likely that projections from DRN cells contribute to the serotonergic innervation of the ventricular plexus, as evidenced by the presence of extensive ventricular plexus terminal degeneration subsequent to microinjection of 5,7-DHT into the DRN. Likewise, retrograde tracing combined with 5-HT immunofluorescence staining has demonstrated that 5-HT-positive cells in DRNdm and DRNvm send projections to the third, fourth, and lateral ventricles (Larsen et al., 1996; Mikkelsen et al., 1997; Simpson et al., 1998).

### 6.5.3 Innervation of Locus Coeruleus by Serotonergic Projections from DRN

There is extensive evidence demonstrating that the LC is targeted by serotonergic projections. Autoradiography has revealed the presence of varicose fibers in the LC that take up [<sup>3</sup>H]5-HT (Leger and Descarries, 1978). It has also been shown that the LC is densely innervated by 5-HT-immunoreactive (Steinbusch, 1981; Van Bockstaele, 2000; Lerger et al., 2001) and SERT-immunoreactive (Sur et al., 1996) fibers. Importantly, the serotonergic fibers innervating the LC are known to make synaptic contact with TH-positive (*i.e.*, catecholamine-containing) dendrites (Van Bockstaele, 2000). Activation of 5-HT receptors alters the activity of LC neurons (Aston-Jones et al., 1991*a*; Charlety et al., 1993; Szabo and Blier, 2001). There is evidence that a substantial portion of the 5-HT released within the LC originates from DRN projections. The extracellular concentration of 5-HT in the LC is increased by electrical or chemical stimulation of the DRN, and is strongly reduced by thermal DRN lesions and by intra-DRN microinjection of 5,7-DHT (Kaehler et al., 1999; Hajés-Korcsok and Sharp, 2002). Microinjection of 8-OH-DPAT, an agonist at somatodendritic 5-HT<sub>1A</sub> autoreceptors that inhibits the firing of serotonergic cells, directly into the DRN also decreased the release of 5-HT in the LC (Kaehler et al., 1999). These findings are consistent with the fact that approximately 50% of the DRN neurons projecting to the LC are 5-HT-immunoreactive (Kim et al., 2004).

After injection of 5,7-DHT into the DRN, degenerating axon terminals were observed in the LC. This finding provides confirmation that serotonergic DRN cells project to the LC. Further, some of the degenerating terminals in the LC could be traced to the border of the neighboring ventricular plexus, indicating that projections from serotonergic DRN neurons descend to the LC within the ventricular plexus. However, there is evidence that the ventricular plexus is not the only fiber pathway through which LC-targeting serotonergic DRN projections descend. Anterograde tracing in normal animals has shown (see Chapter 4.0) that the descending mlf pathway is the source of large caliber fibers—putative serotonergic fibers that are lost after treatment with 5,7-DHT (see Chapter 5.0)—that innervate the LC from DRN. Although the presence of heavy caliber serotonergic DRN axons within LC was not revealed by silver staining, given the fact that relatively long survival times are typically required for detection of degeneration in parent axons, this negative finding was not unexpected. Therefore, it appears that serotonergic DRN projections descend to the LC within the ventricular plexus.

There is long-standing evidence that 5-HT and norepinephrine (NE) receptors act to co-modulate the firing activity of cells in a number of brain regions. For example, both 5-HT and NE facilitate glutamate-induced excitation of facial and spinal motoneurons when applied by microiontophoresis (McCall and Aghajanian, 1979; White and Neuman, 1980). 5-HT and NE increase the frequency of spontaneous excitatory postsynaptic currents (EPSCs) in layer V pyramidal cells of the medial prefrontal cortex (Marek and Aghajanian, 1999), and activation of 5-HT<sub>2A</sub> receptors and  $\alpha_{1B}$ -adrenoceptors induces inhibitory postsynaptic potentials (IPSPs) in layer II pyramidal cells of rat piriform cortex by exciting a subpopulation of GABAergic interneurons (Gellman and Aghajanian, 1993; Marek and Aghajanian, 1996*a*,*b*). It is also recognized that 5-HT and NE enhance the hyperpolarization-activated cationic current I<sub>h</sub> in thalamocortical neurons, promoting a shift from rhythmic burst firing to single spike activity (Pape and McCormick, 1989). Schuerger and Balaban (1993, 1999) have shown that the distribution of noradrenergic fibers in the vestibular nuclei in rats is greatest in LVN and SVN; the present findings demonstrate that these two regions of the vestibular nuclei are also innervated by serotonergic projections originating from DRN. In light of the fact that LC and DRN projections are sources of overlapping vestibular terminal fields, it is possible that noradrenergic projections from the LC and serotonergic projections from the DRN may act to co-modulate the activity of neurons in SVN and dorsal LVN. Cells in dorsal LVN participate in vestibulo-spinal reflexes and send excitatory projections to ipsilateral limb extensor motoneurons via the lateral vestibulo-spinal tract (Wilson and Melvill Jones, 1979; Leong et al., 1984). Electrophysiological experiments indicate that both 5-HT and NE can influence the firing of vestibulo-spinal tract relay neurons in LVN. Microiontophoretic application of 5-HT into the LVN of urethane anesthetized rats enhanced the electromyographic responses of proximal limb extensor muscles while inhibiting the responses of flexor muscles, effects that were attenuated by simultaneous application of the 5-HT<sub>2A/2C</sub> antagonist ketanserin (Li Volsi et al., 1998). Likewise, Licata and coworkers (Licata et al., 1990) examined the effect of 5-HT on cells in rat LVN that project through the lateral vestibulo-spinal tract, and found that microiontophoresis of 5-HT evoked a long-lasting enhancement of the background firing rate in 97% of the units. The ability of 5-HT to stimulate the maintained activity of these LVN neurons was attenuated in the presence of metergoline, methysergide or ketanserin, indicating that the excitation is mediated by 5-HT<sub>2</sub> receptors. Furthermore, in slightly less than half of the units, the excitation produced by 5-HT was preceded by a transient reduction of firing; 5-HT<sub>1</sub> receptors may be responsible for this inhibitory effect because it was also evoked by the nonselective 5-HT<sub>1</sub>/5-HT<sub>2</sub> agonist

5-MeO-DMT but was not blocked by the 5-HT<sub>2</sub> antagonist methysergide. The same workers (Licata et al., 1993*b*) also reported that in rats the spontaneous activity of lateral vestibulo-spinal tract relay neurons in LVN is reduced by iontophoresis of NE, an effect that appeared to be mediated by  $\alpha_2$ -adrenoceptors as it was mimicked by clonidine and antagonized by yohimbine. Conversely, the majority of LVN neurons in decerebrate cats are excited by NE (Yamamoto, 1967; Kirsten and Sharma, 1976). Thus, there is evidence that NE and 5-HT can alter the firing properties of LVN cells involved in vestibulo-spinal reflexes. Finally, given that activation of  $\alpha_2$ -adrenergic heteroreceptors present on 5-HT terminals in neocortex (Feuerstein et al., 1993; Trendelenburg et al., 1994) and hippocampus (Mongeau et al., 1993) has been shown to decrease 5-HT release, it is possible that NE may act to gate the serotonergic input to the vestibular nuclei.

### 6.5.4 Projections from Serotonergic DRN Neurons Innervate the Vestibular Nuclei

Extensive immunohistochemical data demonstrates that the vestibular nuclei are innervated by serotonergic fibers (Steinbusch, 1981, 1991; Harvey et al., 1993; Halberstadt and Balaban, 2003). Retrograde tracing experiments (Halberstadt and Balaban, 2003; Chapter 3.0) have revealed that the serotonergic innervation of the vestibular nuclei arises in large part from cells in the DRN; the present anterograde tracing study provides important confirmation that the DRN sends a sizable serotonergic projection to the vestibular nuclei. Indeed, after injection of 5,7-DHT into the DRN, argyrophilic terminals were present throughout SVN, LVN and MVN. Conversely, few silver-impregnated terminals were found in IVN. These findings parallel those of our retrograde tracing studies which found that relatively few serotonergic DRN cells were labeled by injection sites involving IVN. It was common to find, in animals given intra-DRN injections of 5,7-DHT, that degenerating fibers coursed into MVN from the ventricular plexus. This observation is noteworthy given that the ventricular plexus is known to supply 5-HT- and SERT-immunopositive fibers to MVN (Halberstadt and Balaban, 2003; see also Figure 1.2). In light of these findings, it would appear that serotonergic fibers descending in the ventricular

plexus from the DRN supply innervation to the vestibular nuclei. Thus, several lines of evidence indicate that serotonergic DRN projections to the vestibular nuclei descend (1) within the ventricular plexus (this study), and (2) within the mlf (see Section 5.5.2). Although the functional importance of organizing serotonergic DRN-to-vestibular nucleus projections into two discrete descending pathways is unclear, it may be significant that the two projections have somewhat differing termination patterns, with the latter pathway contributing innervation to the greatest extent to lateral aspects of the vestibular nuclei (SVN, LVN, and lateral parts of MVN). Another organizational feature of serotonergic DRN-to-vestibular nucleus projections is that this pathway involves both fine caliber and heavier caliber fibers. As was shown in Chapters 4.0 and 5.0, our BDA anterograde tracing work indicates that the heavier caliber serotonergic DRN projections descend to the vestibular nuclei exclusively within the mlf pathway. The present experiments extend those findings by demonstrating that the fine caliber serotonergic DRN projections descend to the vestibular nuclei within the ventricular plexus (see Figures 6.6C and 6.7B). Furthermore, two observations serve to confirm that the innervation of the vestibular nuclei by serotonergic DRN projections involves both fine caliber and heavier caliber fibers. First, a mixture of fine and heavier caliber degenerating terminals was present within the vestibular nuclei after microinjection of 5,7-DHT into DRN (see Figures 6.6 and 6.7). Second, in control animals, both fine and heavier caliber 5-HT-immunoreactive fibers are found within the vestibular nuclei (see Figure 6.2A).

A number of electrophysiological studies have shown that activation of 5-HT receptors can modulate neuronal firing in SVN (Licata et al., 1993*a*; Li Volsi et al., 2001), MVN (Licata et al., 1991; Johnston et al., 1993; Amono et al., 2001; Jeong et al., 2003, 2005), and LVN (Licata et al., 1990; Kishimoto et al., 1991, 1994; Li Volsi et al., 2001), and the present anatomical findings demonstrate that the DRN contributes serotonergic projections to those regions of the vestibular nuclei. Further, the results of the present study, as well as those of our other anterograde tracing experiments (see Chapters 4.0 and 5.0), reveal that these serotonergic DRN projections are organized into distinct terminal fields within the lateral aspect of the vestibular nuclei. This is in contrast to the nonserotonergic DRN projections, which are not segregated into discrete terminal regions (discussed in Section 5.5.2). The fact that serotonergic

DRN innervation of the vestibular nuclei is segregated in discrete terminal fields indicates that this projection may be organized to selectively modulate processing within particular vestibular pathways. In particular, the location of the serotonergic terminal fields within the vestibular nuclei indicates that these projections are organized to influence processing in vestibular regions involved in eye movement, velocity storage, vestibular-autonomic interactions, and postural responses.

### 6.5.5 Functional Significance of Serotonergic DRN Projections to Vestibular Nuclei

A number of clinical observations indicate that 5-HT is involved regulating vestibular function, and may play an important role in modulating sensitivity to motion sickness. First, activation of 5-HT receptors, either directly (by administration of 5-HT agonists) or indirectly (by increasing synaptic levels of 5-HT) has been shown to reduce symptoms of vestibular dysfunction in a number of clinical populations. It was recently demonstrated by Marcus and Furman (2006) that patients with migrainous vertigo are much less susceptible to motion sickness after administration of the 5-HT agonist rizatriptan. Further, chronic administration of SSRIs, a treatment known to enhance serotonergic neurotransmission (Chaput et al., 1988; Blier et al., 1990; Davidson and Stamford, 1997; Haddjeri et al., 1998), improves balance function and reduces symptoms of dizziness and vertigo in patients with migraine (Johnson, 1998), chronic subjective dizziness (Staab et al., 2004; Staab and Ruckenstein, 2005), anxiety (Perna et al., 2003; Simon et al., 2005), and in patients with and without major psychiatric symptoms (Staab et al., 2002). Conversely, there is evidence that diminished serotonergic transmission can provoke vestibular dysfunction in otherwise normal individuals. For example, it has been demonstrated that depletion of 5-HT (by administration of a tryptophan-free diet) increases the ability of illusionary visual movement to provoke motion sickness in volunteers (Drummond, 2005). The existence of a relationship between diminished serotonergic transmission and vestibular dysfunction is also supported, albeit indirectly, by the fact that dizziness and vertigo are prominent symptoms of SSRI withdrawal (Black et al., 1993; Coupland et al., 1996; Zajecka et al., 1997). The facilitation of serotonergic neurotransmission that results from chronic 5-HT re-uptake blockade by SSRIs is accompanied by a number of functional adaptations, including desensitization of postsynaptic 5-HT receptors (Kennett et al., 1994; Li et al., 1997; Raap et al., 1999; Yamauchi et al., 2004, 2006; El Manasri and Blier, 2005). It is likely that after discontinuation of chronic SSRI treatment there is a decline of synaptic 5-HT levels due to increased 5-HT re-uptake; the resultant synaptic concentrations of 5-HT may not be sufficient to activate desensitized postsynaptic 5-HT receptors. Therefore, the dizziness and vertigo associated with the SSRI discontinuation syndrome may be a vestibular manifestation of a deficiency of 5-HT transmission. It is tantalizing that in one reported case the SSRI withdrawal syndrome was markedly exacerbated by buspirone (Carrazana et al., 2001), a drug that inhibits the firing of serotonergic DRN neurons by activating somatodendritic 5-HT<sub>1A</sub> autoreceptors (Wilkinson et al., 1987; Fornal et al., 1994), and thus it is possible that during SSRI withdrawal the administration of buspirone results in further attenuation of serotonergic transmission.

The findings of this study demonstrate that serotonergic DRN projections innervate the vestibular nuclei. Hence, the ability of serotonergic transmission to influence vestibular function probably involves the vestibular nuclei. The contention that 5-HT acts within the vestibular nuclei to modulate sensitivity to motion sickness is supported by the findings of several electrophysiological studies which indicate that the responsiveness of neurons in the vestibular nuclei is reduced by activation of 5-HT receptors. For example, one group (Amano et al., 2001) reported that the ability of lateral tilt stimulation to activate  $\alpha$ - and  $\beta$ -type neurons in the MVN of rats anesthetized with chloral hydrate is attenuated by iontophoresis of the 5-HT<sub>1A</sub>-selective agonist tandospirone. Importantly, this effect was reduced in the presence of the 5-HT<sub>1A</sub> antagonist WAY-100,635. Further, in  $\alpha$ -chloralose-anesthetized cats, electrical stimulation of DRNvm has been shown to decrease the ability of vestibular nerve stimulation to activate LVN neurons that project to the abducens nucleus (Kishimoto et al., 1991). A similar effect was produced by iontophoresis of 5-HT, and the inhibition induced by application of 5-HT and by DRN stimulation was reduced by the 5-HT antagonist methysergide. The same workers later reported (Kishimoto et al., 1994) that identical inhibitory effects were evoked by iontophoresis of the 5-HT<sub>1A</sub> agonist 8-OH-DPAT.

Moreover, microiontophoresis of 5-HT and 8-OH-DPAT was also found to inhibit the excitation of LVN neurons by glutamate, which is one of the neurotransmitters of the vestibular nerve (reviewed by Darlington et al., 1995). Another group has also examined the effect of 5-HT on the response of vestibular nucleus neurons to glutamate (Li Volsi et al., 2001). Recording primarily from LVN and SVN in urethane-anesthetized rats, these workers found that the most common response to 5-HT was a reduction in glutamate-evoked responses, although in a minority of cells the response to glutamate was facilitated. The inhibitory effect of 5-HT was diminished by the 5-HT<sub>2A</sub> antagonist ketanserin, whereas 8-OH-DPAT enhanced glutamate-induced excitation. Taken together, these electrophysiological findings demonstrate that the responsiveness of vestibular nucleus neurons is typically attenuated by activation of 5-HT receptors.

The fact that dizziness and vertigo occurring during the SSRI discontinuation syndrome are exacerbated by movement, especially of the head and eyes (Pyke, 1995; Coupland et al., 1996), may reflect an elevated sensitivity of vestibular pathways during SSRI withdrawal. It has been proposed that serotonergic DRN projections are involved in coordinating sensory processing with motor function (Jacobs and Fornal, 1993), and a major tenet of this hypothesis is that 5-HT acts to suppress sensory information processing during motor output. As the vestibular nuclei are a site of sensorimotor integration for several postural, ocular, autonomic and ascending sensory pathways, this hypothesis suggests that serotonergic input from the DRN may be important for regulating the expression of vestibular-evoked motor responses to both self-imposed and externally imposed motion. As was noted above, studies indicate that the activation of 5-HT receptors decreases the evoked-responses of vestibular nucleus neurons (Kishimoto et al., 1991, 1994; Amano et al., 2001; Li Volsi et al., 2001). Therefore, failure of serotonergic DRN projections to actively modulate the responsiveness of vestibulo-spinal, vestibulo-collic, and/or vestibuloocular pathways during ongoing head and eye movements may explain why SSRI withdrawal is intensified by motor activity.

### 7.0 GENERAL DISCUSSION

## 7.1 ORGANIZATION OF PROJECTIONS FROM DORSAL RAPHE NUCLEUS TO THE VESTIBULAR NUCLEI

We have previously demonstrated that the DRN projects heavily to the vestibular nuclei (Halberstadt and Balaban, 2003); the objective of the present investigation was to fully characterize the organization and termination patterns of this projection in Long-Evans rats. To accomplish that goal, we made use of established retrograde (Chapter 3.0) and anterograde (Chapter 4.0) tracing techniques, as well as two anterograde tracing techniques that can distinguish between serotonergic and nonserotonergic DRN projections (Chapter 5.0 and Chapter 6.0, respectively). The results of these experiments confirmed the major findings of our initial study: (1) that the vestibular nuclei are innervated by DRN projections, and (2) that these projections originate from both serotonergic and nonserotonergic DRN neurons, with at least a quarter of the DRN cells projecting to the vestibular nuclei being nonserotonergic (see Chapter 3.0). These experiments also confirmed that regional differences exist in the termination patterns of DRN projections within the vestibular nuclei, with the innervation typically being heavily enriched in the rostral and medial aspects of the vestibular nuclei and very sparse in IVN. Additionally, it was observed that substantial differences exist in the terminal distribution of serotonergic and nonserotonergic DRN projections within the vestibular nuclei. Indeed, it appears that the serotonergic and nonserotonergic DRN projections to the vestibular nuclei represent distinct DRN projection systems, at least in terms of their organization. Regarding the organizational features of these two projection systems, our experiments specifically revealed the following:

# 7.1.1 Serotonergic and Nonserotonergic DRN Projections Terminate Differentially Within the Vestibular Nuclei

Comparing the results of the three anterograde tracing studies reported in Chapters 4.0, 5.0, and 6.0, it is apparent that the termination patterns of the serotonergic and nonserotonergic DRN projections within the vestibular nuclei is dissimilar. Anterograde tracing of all DRN projections (Chapter 4.0) demonstrated that these projections terminate over the entire rostrocaudal extent of the vestibular nuclei, within SVN, MVN, and LVN. Selective anterograde tracing of nonserotonergic DRN projections (Chapter 5.0) revealed that these projections ramify extensively within rostral SVN and the medial aspect of MVN that borders the fourth ventricle, whilst terminating more sparsely within lateral regions of the vestibular nuclei—in particular ventrolateral MVN, caudal SVN, and LVN. In contrast to the predominantly medial distribution of the nonserotonergic fibers, selective anterograde tracing of nonserotonergic DRN projections (Chapter 6.0) revealed that these projections ramify within both the medial and lateral aspects of the vestibular nuclei. Interestingly, neither serotonergic nor nonserotonergic DRN projections provided substantial terminal innervation to IVN. Taken together, these findings indicate that the vestibular nuclei can be divided into three sub-regions based on the type of DRN input received: (1) a region that involves rostral SVN and medial MVN and is heavily targeted by both serotonergic and nonserotonergic DRN projections; (2) a region that involves caudal SVN, lateral MVN, and LVN and is more heavily innervated by projections from serotonergic DRN cells than by projections from nonserotonergic DRN cells; and (3) IVN, which is only weakly targeted by projections from DRN.

## 7.1.2 Serotonergic and Nonserotonergic DRN Projections Terminate Within Three Vestibular Terminal Fields

The results of our anterograde tracing studies demonstrate that DRN projections terminate within three distinct, although overlapping, terminal regions within the vestibular nuclei. The anatomical localization

of these three terminal regions—the periventricular field, the rostrodorsal field, and the caudoventral field—is illustrated in Figure 7.1. It appears that the nonserotonergic and serotonergic DRN projections contribute differentially to these three vestibular terminal regions.

The periventricular vestibular terminal field involves SVN and the region of the vestibular nuclei that borders the fourth ventricle (see Figure 7.1), and receives both serotonergic and nonserotonergic DRN innervation. We have demonstrated, using an anterograde tracing technique that selectively labels nonserotonergic fibers (Chapter 5.0), that projections from nonserotonergic DRN cells are fine in caliber and descend to the vestibular nuclei exclusively within the ventricular fiber plexus, contributing fine caliber innervation primarily to SVN and periventricular regions of the vestibular nuclei. Likewise, by selectively tracing the degenerating processes of serotonergic DRN cells (Chapter 6.0), we were able to demonstrate that fine caliber serotonergic fibers descend within the ventricular plexus and ramify within rostral SVN and the periventricular aspect of medial MVN. Thus, the periventricular vestibular terminal field is targeted by fine caliber serotonergic and nonserotonergic fibers that descend from the DRN within the ventricular plexus.

It appears that nonserotonergic DRN projections to the vestibular nuclei terminate primarily within the periventricular terminal field, with regions of the vestibular nuclei located further laterally being more sparsely innervated. Indeed, the results of our retrograde tracing experiment (Chapter 3.0) indicate that compared with the medial aspect of the vestibular nuclei, the lateral aspect of the vestibular nuclei receives a greater proportion of DRN input from serotonergic neurons than from nonserotonergic neurons. The later vestibular injection case 2005-19 labeled a total of 38 (single- and double-labeled) 5-HT positive DRN cells and 15 5-HT negative DRN cells (72% 5-HT positive), whereas more medial and caudal injections (R6 and R9) produced 56 or 76 5-HT positive and 44 or 50 5-HT negative cells, respectively (56% or 60% 5-HT positive). Similar proportions of 5-HT-immunopositive DRN cells were found to project to the other caudal/medial cases, including T1 (63%), T3 (59%), and T4 (61%). These findings support our contention that nonserotonergic DRN innervation is concentrated within the periventricular terminal field.



## Figure 7.1. Illustration of the location of the periventricular, rostrodorsal, and caudoventral DRN terminal fields within the vestibular nuclei.

The periventricular terminal field is targeted by fine caliber serotonergic and nonserotonergic fibers that descend from the DRN within the ventricular plexus. The rostrodorsal terminal field and the caudoventral terminal field are targeted by heavier caliber serotonergic fibers that descend from the DRN within the medial longitudinal fasciculus (mlf). (Abbreviations: g7, genu of facial nerve; IVN, inferior vestibular nucleus; LC, locus coeruleus; LVN, lateral vestibular nucleus; NTS, nucleus tractus solitarius; PrH, nucleus prepositus hypoglossi; SVN, superior vestibular nucleus.

In addition to the presence of serotonergic innervation within the periventricular field, serotonergic DRN innervation of the vestibular nuclei is also distributed in discrete rostrodorsal and caudoventral terminal regions that are anterogradely labeled with BDA in normal animals (Chapter 4.0) but not in animals pretreated with 5,7-DHT to destroy the serotonergic projection system (Chapter 5.0). This finding clearly indicates that the DRN projections innervating the latter two fields are predominantly serotonergic. The presence of serotonergic DRN terminal innervation within these two terminal regions was confirmed by selectively tracing the degenerating processes of serotonergic DRN cells (Chapter 6.0). Although it appears that serotonergic DRN cells contribute innervation to all three vestibular terminal regions, the morphology and course of descent of the serotonergic fibers innervating the periventricular field. In particular, the former two fields are innervated by heavier caliber serotonergic fibers that descend from the DRN exclusively within the mlf pathway, in contrast to the fine caliber innervation of the latter field that descends in the ventricular plexus.

The anatomical localization of the rostrodorsal and caudoventral terminal regions is illustrated in Figure 7.1. The rostrodorsal serotonergic terminal region involves ventral SVN, the dorsal pole of the rostral two-thirds of MVN, and dorsal LVN. Conversely, the caudoventral serotonergic terminal region involves the ventral pole of mid-level MVN, all of caudal MVN, and the ventral part of caudal LVN. In addition to their localization within the vestibular nuclei, a second major difference between these two serotonergic terminal fields is that the innervation of the rostrodorsal terminal region arises predominantly from ipsilateral DRN whereas both ipsilateral and contralateral DRN supply innervation to the caudoventral terminal field.

The rostrodorsal and caudoventral terminal fields are innervated by heavier caliber serotonergic fibers, whereas the periventricular terminal field is innervated by fine caliber serotonergic fibers. One consequence of this organization may be that the principal mode of serotonergic transmission within the lateral and medial regions of the vestibular nuclei is different. It has been reported that heavier caliber 5-HT-immunopositive fibers do not co-localize SERT (Brown and Molliver, 2000; Nielsen et al., 2006).

Indeed, the fact that we were able to demonstrate that heavier caliber serotonergic fibers are present in lateral regions of the vestibular nuclei using 5-HT immunostaining (see Figure 6.2), but not using SERT immunostaining (Halberstadt and Balaban, 2003), argues that the heavier caliber serotonergic fibers that innervate the lateral aspect of the vestibular nuclei do not express SERT. There is extensive evidence that there is a volume or paracrine component to serotonergic transmission (Bunin and Wightman, 1998, 1999; Zhou et al., 1998), and the presence of SERT on extrasynaptic axon segments indicates that SERT may act to regulate 5-HT volume transmission (Zhou et al., 1998). As such, the absence of SERT within the heavier caliber serotonergic innervation that is present laterally within the vestibular nuclei raises the possibility that the action of 5-HT within that region of the vestibular nuclei is much more diffuse and nonspecific than in the medial aspect of the vestibular nuclei where SERT is heavily expressed (see Figure 1.2). Indeed, the presence of SERT would tend to limit the distance to which 5-HT could diffuse from synapses within the periventricular field.

## 7.1.3 Serotonergic and Nonserotonergic DRN Cells Give Rise to Anatomically Distinct Projection Systems

Our anterograde tracing experiments reveal that two major differences exist in the organization of serotonergic and nonserotonergic projections from DRN to the vestibular nuclei: (1) nonserotonergic projections descend within the ventricular plexus, whereas serotonergic projections descend within both the ventricular plexus and the mlf pathway, and (2) the projections terminate differently within the vestibular nuclei, with serotonergic DRN neurons providing innervation to both the medial and lateral aspects of the vestibular nuclei, and nonserotonergic DRN neurons projecting primarily to SVN and medial MVN. Taken together, these findings indicate that the serotonergic and nonserotonergic DRN projections actually represent distinct projection systems. Although it has been known for some time that DRN projections arise from serotonergic and nonserotonergic cells, very few previous studies have examined the comparative distribution of these projections within individual target regions. After

injecting PHA-L into the DRN of two rats, Kosofsky and Molliver (1987) used 5-HT immunofluorescence staining to compare the organization of serotonergic and nonserotonergic DRN projections within regions of cerebral cortex. They reported that anterogradely labeled 5-HT-positive and 5-HT-negative axons were found clustered together in cortex and that only 5-20% of the axons were nonserotonergic. A similar study was conducted by Aznar et al. (2004), using a combination of 5-HT immunofluorescence staining and injections of BDA into the DRN. They found that 86% of the BDAlabeled fibers in the lateral septum, 90% of the BDA-labeled fibers in the medial septum, and 83% of the BDA-labeled fibers in the diagonal band of Broca were nonserotonergic; both the serotonergic and nonserotonergic fibers projected onto cabindin- and parvalbumin-positive cells within the septum. Taken together, these findings indicate that serotonergic and nonserotonergic DRN projections are often colocalized in target regions, although the density to which these two projections terminate within target regions is not interrelated. Nonetheless, our studies demonstrate that within at least one particular target region there is not complete overlap between these two projections. Therefore, rather than treating the DRN as a single projection system containing both serotonergic and nonserotonergic components, it may be more accurate to treat it as two separate projection systems that originate from within the same nucleus.

Even though it appears that serotonergic and nonserotonergic DRN cells give rise to distinct projection systems, it is likely that there are extensive interactions between these two projections. There is evidence that serotonergic and nonserotonergic neurons interact within the DRN. Electrophysiological evidence indicates that glutamatergic DRN cells (Jolas and Aghajanian, 1997; Liu et al., 2002*a*) and GABAergic DRN cells (Liu et al., 2000, 2002*b*) send projections to serotonergic DRN neurons. Conversely, although it was reported initially that the only DRN neurons that express  $5-HT_{1A}$  receptor immunoreactivity are serotonergic (Sotelo et al., 1990), more recent findings have revealed that nonserotonergic DRN neurons also express  $5-HT_{1A}$  receptors (Kirby et al., 2003; Day et al., 2004). Indeed, several recent *in vitro* electrophysiological studies have demonstrated that 5-HT- and tryptophan hydroxylase-immunonegative DRN neurons respond to bath application of  $5-HT_{1A}$  agonists (Kirby et al.,

2003; Beck et al., 2004; Marinelli et al., 2004). Furthermore, most GABAergic DRN neurons express 5- $HT_{2c}$  receptor mRNA (Serrats et al., 2005), and there is evidence that the selective 5- $HT_{2A/2C}$  agonist DOI (1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane) can excite putative GABAergic DRN neurons (Liu et al., 2000; see also Marinelli et al., 2004). Evidence has also been presented which indicates that GABAergic and serotonergic DRN neurons are reciprocally interconnected (Bagdy et al., 2000). These findings indicate that there is substantial mutual feed-forward regulation between serotonergic and nonserotonergic DRN. To our knowledge the question of whether there are similar interactions between serotonergic and nonserotonergic DRN fibers in target regions has not been explored, although it is possible that this type of processing may occur in regions where these projections substantially overlap.

## 7.1.4 DRN Neurons Projecting to the Vestibular Nuclei Also Target the Origin of the Coeruleo-Vestibular Tract

The caudoventral aspect of the LC supplies noradrenergic innervation to LVN and SVN via a coeruleovestibular projection pathway (Schuerger and Balaban, 1993, 1999). Our BDA anterograde tracing experiment (Chapter 4.0) demonstrated that many labeled DRN fibers that ramify within SVN and other lateral aspects of the rostrodorsal vestibular terminal region also supply innervation to caudoventral LC. Neurochemically-selective anterograde tracing methods (Chapters 5.0 and 6.0) revealed that this innervation arises from both nonserotonergic and serotonergic DRN neurons, as was reported earlier by a retrograde tracing study (Kim et al., 2004). These findings raise the possibility that in addition to the existence of direct projections from the DRN to the vestibular nuclei, the DRN may also influence processing in the vestibular nuclei indirectly, by altering the activity of the LC and the coeruleo-vestibular pathway. Indeed, there is evidence that both serotonergic and nonserotonergic DRN projections can influence the firing activity of LC neurons (Hajés-Korcsok and Sharp, 1996, 2002). LC neurons are activated during states of alertness and vigilance and in response to novel stimuli (Aston-Jones et al., 1984, 1991*b*; Foote et al., 1991), and one function of the coeruleo-vestibular pathway may be to alter vestibulo-spinal reflex sensitivity (Pompeiano et al., 1991; Schuerger and Balaban, 1993) according to ongoing situational demands. Therefore, DRN projections may influence vestibulo-spinal reflex sensitivity through a direct action in the vestibular nuclei (Licata et al., 1990; Li Volsi et al., 1998) as well as indirectly through the coeruleo-vestibular pathway.

## 7.1.5 Serotonergic and Nonserotonergic DRN Neurons Projecting to the Vestibular Nuclei Send Collateralized Projections to Central Amygdaloid Nucleus

Dual injections of retrograde tracers into the vestibular nuclei and CeA revealed that approximately 30% of the serotonergic neurons and approximately 23% of the nonserotonergic neurons that project to the vestibular nuclei also project, via axon collaterals, to CeA (Chapter 3.0). This subset of DRN projections to the vestibular nucleus may be selectively organized to coordinate processing within the ascending vestibulo-parabrachio-forebrain pathway, a network implicated in the linkage between balance disorders and anxiety disorders. Indeed, we have previously theorized that these collateralized projections may exist to co-modulate vestibular and affective responses (Halberstadt et al., 2001; Balaban, 2002).

It is well established that both serotonergic and nonserotonergic DRN cells send collateralized projections to functionally related target nuclei (van der Kooy and Kuypers, 1979; van der Kooy and Hattori, 1980; de Olmos and Heimer, 1980; Köhler and Steinbusch, 1982; Imai et al., 1986; Van Boeckstaele et al., 1993; Kirifides et al., 2001). Studies have demonstrated that several nuclei within the ascending vestibulo-PBN-forebrain pathway receive collateralized axonal projections from DRN. Petrov and colleagues have shown that single DRN neurons send projections to both PVN and lateral PVN (Petrov et al., 1992), and these investigators later found that DRN cells send collateralized projections to PVN and CeA (Petrov et al., 1994). Based on the present retrograde tracing data, it is now known that the vestibular nuclei and CeA are also targeted by collateralized DRN projections. Thus, the results of our retrograde tracing experiments confirm and extend the previous findings regarding the organization of

DRN projections to the ascending vestibulo-PBN-forebrain pathway. It now appears that all levels of the vestibulo-PBN-forebrain pathway are targeted by collateralized DRN projections, and it is likely that this is an important organizational principle governing how processing in the ascending pathway is regulated by input from the DRN. Similar findings have been reported for the trigeminal somatosensory pathway (Kirifides et al., 2001), which involves the trigeminal nucleus, ventral posterior medial thalamus, and primary somatosensory cortex. Further, it is possible that certain DRN cells may send projections to three (or more) component nuclei of the vestibulo-PBN-forebrain pathway, although this question has not been specifically addressed using retrograde tracing with three tracers. It has been shown previously that a small number of cells in the ventral part of DRN send collateralized projections to the septum, medial thalamus, and olfactory cortex (de Olmos and Heimer, 1980). We found that approximately 28% of the DRN cells projecting to CeA also project to PVN (Petrov et al., 1994), it is possible that at least ~2–3% of the DRN cells projecting to the vestibular nuclei also send projections to CeA and PVN. This theoretical population of DRN neurons could act to coordinate processing in the input (vestibular nuclei), intermediate (PBN), and output (forebrain) regions of the ascending pathway.

### 7.2 CONCLUSIONS

These studies have revealed a number of previously unknown organizational features of the projection from DRN to the vestibular nuclei and related structures; these features are illustrated schematically in Figure 7.2. These studies have demonstrated that projections from serotonergic and nonserotonergic DRN neurons heavily innervate the vestibular nuclei, with the serotonergic innervation organized into discrete rostrodorsal, caudoventral, and periventricular vestibular terminal fields. The DRN also targets the caudal LC, which in turn provides noradrenergic input to lateral and rostral aspects of the vestibular nuclei which are also targeted by the rostrodorsal terminal field. Lastly, some of the serotonergic and



## Figure 7.2. Schematic diagram illustrating the organization of DRN projections to the vestibular nuclei and related structures.

The DRN sends projections to the vestibular nuclei, which terminate within rostrodorsal, caudoventral, and periventricular terminal fields. The DRN also projects to locus coeruleus, which in turn gives rise to noradrenergic projections that terminate with aspects of the rostrodorsal DRN terminal field. A subset of DRN cells that project to the vestibular nuclei also send collateralized projections to the central amygdaloid nucleus. Connections between the vestibular nuclei and components of the vestibulo-parabrachio-forebrain network are also illustrated.

nonserotonergic DRN neurons projecting to the vestibular nuclei also project to CeA, a component of a vestibulo-PBN-forebrain network that also involves the hypothalamus and regions of cortex.

These studies further indicate that there may be major differences in the anatomical organization of the serotonergic and nonserotonergic projections from DRN, both in their course, morphology, and termination patterns. Although little is known about the function of the nonserotonergic DRN projections to the vestibular nuclei, there is evidence that the serotonergic projections may act to modulate the responsiveness of neurons in the vestibular nuclei to self-imposed and externally imposed motion, and may influence motion sickness sensitivity.

Based on these findings, there are a number of important issues that require further investigation. One significant issue that needs to be examined is the identity of the transmitter substance(s) present in the nonserotonergic DRN projections to the vestibular nuclei; without this information it is not possible to understand the functional importance of this projection. Another issue is that very little is actually known about the distribution of 5-HT receptor subtypes within the vestibular nuclei, including the comparative regional distribution and also the ultrastructural localization of these receptors. Importantly, given that there is currently a large amount of work being done to develop serotonergic agents as clinical treatments for vestibular dysfunction (e.g., Marcus et al., 2005; Marcus and Furman, 2006), information regarding the type and distribution of 5-HT receptors within the vestibular nuclei would provide insight into which types of serotonergic ligands are most likely to be clinically useful. Lastly, it needs to be determined whether DRN neurons projecting to the vestibular nuclei also send collateralized projections to PBN, and whether administration of 5-HT receptor agonists and antagonists alters the transmission of vestibular information through the ascending vestibulo-parabrachial pathway in experimental animals. These types of experiments would help to delineate the functional importance of having the DRN exert a coordinated modulation influence on the structures involved in that pathway. In conclusion, although substantial further work will be required to fully understand the significance of DRN-vestibular interactions, the experiments detailed herein have advanced our understanding of the anatomy of the circuitry responsible for these interactions.

### ABBREVIATIONS

ABC	avidin-biotin-peroxidase
AP	anteroposterior
BDA	biotinylated dextran amine
BSA	bovine serum albumin
ССК	cholecystokinin
CeA	central amygdaloid nucleus
CLi	caudal linear raphe nucleus
CRF	corticotropin-releasing factor
СТЬ	cholera toxin subunit B
DAB	diaminobenzidine tetraydrochloride
5,6-DHT	5,6-dihydroxytryptamine
5,7-DHT	5,7-dihydroxytryptamine
DRN	dorsal raphe nucleus
DRNdm	dorsal raphe nucleus, dorsomedial subdivision
DRNI	dorsal raphe nucleus, lateral subdivision
DRNvm	dorsal raphe nucleus, ventromedial subdivision
DY	Diamidino Yellow
EPSCs	excitatory postsynaptic currents
FB	Fast Blue
HRP	horseradish peroxidase
5-HT	serotonin
i.c.v.	intracerebroventricular
IPSPs	inhibitory postsynaptic potentials
IVN	inferior vestibular nucleus
LC	locus coeruleus
LSD	lysergic acid diethylamide

LVN	lateral vestibular nucleus
LVNα	lateral vestibular nucleus pars $\alpha$
LVNβ	lateral vestibular nucleus pars $\beta$
LVNγ	lateral vestibular nucleus pars $\gamma$
MDA	3,4-methylenedioxyamphetamine
MDMA	3,4-methylenedioxymethamphetamine
5-MeO-DMT	5-methoxy-N,N-dimethyltryptamine
MFB	medial forebrain bundle
mlf	medial longitudinal fasciculus
MRN	median raphe nucleus
MVN	medial vestibular nucleus
NE	norepinephrine
6-OH-DA	6-hydroxydopamine
8-OH-DPAT	8-hydroxy-2-(N,N-dipropylamino)tetralin
PAG	periaqueductal grey
PBN	parabrachial nucleus
PBS	phosphate-buffered saline
PCA	para-chloroamphetamine
PHA-L	Phaseolus vulgaris-leucoagglutinin
PLP	paraformaldehyde-lysine-periodate
PrH	nucleus prepositus hypoglossi
PVN	paraventricular nucleus of the hypothalamus
RMg	nucleus raphe magnus
ROb	nucleus raphe obscurus
RPa	nucleus raphe pallidus
SERT	serotonin transporter
SLN	supralemniscal nucleus
SSRI	selective serotonin reuptake inhibitor
SVN	superior vestibular nucleus
TH	tyrosine hydroxylase
ТРН	tryptophan hydroxylase
TRITC	tetramethyl rhodamine isothiocyanate
VIP	vasoactive intestinal polypeptide

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