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Projections from the vestibular nuclei and nucleus prepositus hypoglossi to dorsal raphe nucleus in rats

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Abstract

The serotonergic system regulates processing in components of the vestibular nuclear complex, including the medial vestibular nucleus (MVe) and nucleus prepositus hypoglossi (PH). Recent studies using anterograde and retrograde tracers have shown that vestibular nuclei are targeted by regionally-selective projections from the serotonergic dorsal raphe nucleus. The objective of the present investigation was to determine whether the DRN is targeted by projections from the vestibular nuclear complex in rats, using the anterograde tracer biotinylated dextran amine (BDA). After injection of BDA into PH or the caudal parvocellular division of MVe, labeled fibers and terminals were observed in the ventromedial and lateral subdivisions of DRN. These findings indicate that projections from the vestibular nuclei and PH are organized to modulate processing within specific functional domains of the DRN.

Keywords

vestibular nuclei; nucleus prepositus hypoglossi; dorsal raphe nucleus; biotinylated dextran amine

The largest concentration of serotonergic neurons in the central nervous system (CNS) is found in the dorsal raphe nucleus (DRN). The DRN is located in the midbrain, in the ventral part of the periaqueductal gray (PAG), and it extends caudally into the rostral pons. Subdivisions of the nucleus include the dorsomedial (DRNdm), ventromedial (DRNvm), and lateral (DRNl) cell groups [33]. The DRN has been shown to project widely throughout the brain [35,36], innervating most nuclei of the neuraxis.

5-HT neurons of the DRN have been implicated in a range of behavioural and physiological processes, including feeding, nociception, motor control, sleep, and thermoregulation [11], and DRN cells respond to a variety of sensorimotor stimuli [3,8,30,41]. The DRN neurons receive afferent input from a number of CNS regions, including locus coeruleus and other brainstem catecholaminergic nuclei, medial prefrontal cortex, hypothalamus, habenula, and parabrachial nucleus [16,17,25,26].

During the previous decade, a large amount of preclinical evidence has indicated that the serotonergic system regulates processing in the vestibular nuclei. Recently, it was also shown, using retrograde and anterograde tracing [6,7], that the DRN projects heavily to the vestibular

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nuclei, and the projections from DRN are distributed in distinct rostradorsal and caudoventral terminal fields within the vestibular nuclei. The DRN is often reciprocally interconnected with afferent regions [10,16], and there is some evidence from retrograde tracing studies that components of the vestibular nuclear complex, including nucleus prepositus hypoglossi (PH), project to DRN [13,14,17]. The objective of the present investigation was to determine whether the caudal regions of the vestibular system which are heavily targeted by the caudoventral DRN terminal field—PH and the caudal aspect of the parvicellular division of medial vestibular nucleus (MVePC)—project to subdivisions of the DRN. Therefore, we injected the anterograde tracer biotinylated dextran amine (BDA) into PH and caudal MVePC, and analyzed the distribution of anterograde labelling within DRN.

MATERIALS AND METHODS

All animal experiments were reviewed and approved by University of Pittsburgh Institutional Animal Care and Use Committee, and conformed to the National Institute of Health Guide for the Care and Use of Laboratory Animals. A total of 20 adult male Sprague-Dawley rats (220–280 g, Hilltop Laboratories, Scottsdale, PA) were anesthetized using a mixture of xylazine (6 mg/kg, i.m.), ketamine (50 mg/kg, i.m.) and acepromazine (0.5 mg/kg, i.m.), positioned in a stereotaxic apparatus, and a scalp incision was made from the supraorbital process to the interparietal bone. A burr hole was drilled in the skull, and a 7.5% solution of BDA (10,000 MW; Molecular Probes, Eugene, OR, USA) dissolved in 10 mM phosphate buffer (pH 7.2–7.4) was injected iontophoretically through a glass micropipette (10–50 μ m tip diameter) using 5 μ A positive pulsed current (7 s on/7 s off) for a period of 15–20 min. The BDA injections were targeted toward the dorsomedial part of MVePC, the lateral part of the contralateral SpVe, or caudal PH, using the stereotaxic coordinates of Paxinos and Watson [24]. After completion of the injection the electrode was left in place for 5 min before being withdrawn, the burr hole was closed with bone wax, and the skin overlying the injection site was sutured. The animals were allowed to fully recover from anaesthesia under observation before being returned to the housing facility. Ketoprofen (5 mg/kg, i.m.) was administered post-operatively as an analgesic.

After a survival time of 7 days, the animals were euthanized using sodium pentobarbital (100 mg/kg, i.p.), and perfused transcardially using 300 ml of saline followed by 300 ml of a 4% paraformaldehyde–lysine–periodate fixative solution [19]. Subsequently, the brain was post-fixed for 1 day at 4 °C, and transferred to 30% sucrose in phosphate-buffered saline (PBS, 0.9% NaCl in 50 mM phosphate buffer, pH 7.3) for two days at 4 °C. Transverse frozen sections (35 μ m thick) from the pons and medulla were cut on a freezing microtome. Tissue was collected in six sequential wells, each containing a rostrocaudal series of sections spaced 210 μ m apart, and stored at –20 °C in cryoprotectant until it was processed immunohistochemically for BDA or for BDA and 5-HT.

To visualize BDA labeling, sections were processed using a one-step avidin–biotin–peroxidase procedure [9], and then reacted for 1–2 min with a nickel-enhanced diaminobenzidine tetrahydrochloride solution. 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 solutions, cleared with xylene, and coverslipped with DPX Mountant (Fluka, Milwaukee, WI, USA).

A subset of sections taken from levels spanning the midbrain was stained for both BDA and 5-HT-immunoreactivity. After staining for BDA was completed, the sections were rinsed with PBS and treated overnight with blocking buffer (PBS with 2% bovine serum albumin (BSA) and 0.2% Triton X-100) containing 5% nonfat dry milk. Tissue was rinsed with PBS to remove the nonfat dry milk, treated with avidin/biotin block (Avidin/Biotin Blocking Kit, Vector Laboratories), rinsed with PBS, and incubated for 48 h at 4° C in a rabbit polyclonal anti-5-HT

antibody (1:100,000; ImmunoStar Inc., Hudson, WI, USA) diluted with blocking buffer. Following extensive washing with PBS, the sections were treated with a biotinylated goat anti-rabbit antibody (1:250; Vector Laboratories) diluted in PBS containing 2% BSA. After several washes with PBS, sections were incubated in ABC reagent (Vectastain ABC Elite Kit, Vector Laboratories), washed repeatedly with PBS, and transferred to 50 mM Tris buffer (pH 7.2). 5-HT-immunoreactivity was then visualized by reaction with Vector NovaRed chromagen (Vector NovaRed Substrate Kit, Vector Laboratories). The sections were rinsed with distilled water and mounted on gelatin/chrome alum-subbed slides. After air-drying overnight, sections were dehydrated through a graded series of ethanol, cleared with xylene, and coverslipped with DPX Mountant (Fluka, Milwaukee, WI). Sections were examined using an Olympus BH-2 microscope in order to visualize the distribution of BDA-labeled fibers within the brainstem. Every section which contained anterogradely-labeled terminals was analyzed using camera lucida drawings.

Digital images were obtained using a Nikon Eclipse E600N microscope equipped with a Spot RT Monochrome digital 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 Corporation, Downingtown, PA, USA). Adobe Photoshop 7.01 software (Adobe System, San Jose, CA, USA) was used for brightness and contrast adjustments, and cropping.

RESULTS

The BDA injection sites were centered in the targeted regions with minimal involvement of adjacent structures in 9 animals, and data from only these cases is presented. Of these 9 BDA injection cases, five (cases 1, 2, 3, 6, and 22) involved MVePC, three (cases 21, 25, and 26) involved the PH, and one (case 27) was localized within SpVe. All of the BDA injection sites displayed a dense central core of labeled neuronal somata and fibers surrounded by a peripheral region containing scattered neuronal perikarya with dendrites that extended into the core. Two representative injection sites are illustrated in Figure 1; case 1 (Fig. 1A) was centered within the dorsomedial part of caudal MVePC, and case 25 (Fig. 1B) was centered within the lateral part of rostral PH.

After injection of BDA into the dorsomedial part of caudal MVePC, anterograde labeling was detected within DRN. A mixture of small- and medium-caliber labeled fibers traveled rostrally from MVePC within the mlf, primarily ipsilateral to the injection site. At the level of the DRN, some of the labeled fibers turned dorsally, ramifying predominantly within the ventral aspect of ipsilateral DRN. Ramifications were particularly extensive in the lateral part of DRNvm and the ventral part of DRNI (Figs. 2A, 3A). Conversely, there were relatively few labeled axons within DRNdm and the ventral part of DRNI. At far rostral DRN levels, labeled fibers entered the trochlear nucleus from the mlf; most of these fibers ramified locally, although some of the fibers did extend dorsally into DRN. The anteroposterior distribution of BDA-labeling within the DRN varied with the location of the injection site in MVePC. For example, in cases 3 and 6 where the injection sites were situated more dorsally than medially within MVePC, the anterograde labeling was confined primarily within caudal levels of DRN. By contrast, the labeling was located predominantly within the mid-level and rostral levels of the DRN when the injection sites were located medially in MVePC (cases 1, 2 and 22).

Following injection of BDA into PH, anterogradely-labeled fibers and terminals were observed within all DRN subdivisions (Fig. 2B). Heavier-caliber labeled fibers ascended from the PH injection site within the mlf, primarily ipsilateral to the injection site. These BDA-labeled fibers gave rise to a dense plexus of thin-caliber fibers and terminals within the DRN (Fig. 3B). Although a large number of BDA-labeled fibers extended throughout the mediolateral and

dorsoventral extent of the DRN, the density of the labeled fiber plexus was especially high in the lateral part of DRNvm and the ventral part of DRNI. Nevertheless, the density of the plexus of labeled fibers did vary between cases, depending on the AP level of the PH injection site. In fact, the injection site centered in the rostral aspect of the PH (case 25) produced a lower density of labeling than cases with injections centered more caudally in PH (cases 21 and 26). Interpretation of injection cases 21 and 26 is confounded by the fact that in both animals there was some spread of BDA into the mlf. However, anterograde labeling is not observed within any DRN subdivision after injection of BDA directly into the mlf (Cuccurazzu and Halberstadt, unpublished observations); hence, it is likely that the anterograde DRN labeling observed in cases 26 and 21 originates from caudal PH and not from fibers of passage within the mlf. As was observed with injection sites located in MVePC, the PH injection cases were associated with a dense fiber plexus within the ipsilateral trochlear nucleus.

To verify that the BDA-labeled fibers terminate within the DRN, we used a dual-labeling technique in which a subset of sections from the midbrain of BDA-injected animals were stained for both BDA and 5-HT immunoreactivity. Numerous 5-HT-positive neurons were found within the dorsomedial, ventromedial, and lateral subdivisions of the DRN, throughout the rostrocaudal extent of the nucleus. BDA-labeled terminals were often found interspersed among these 5-HT-immunoreactive DRN neurons (Fig. 3C, D). Conversely, BDA-labeling was not observed in other serotonergic nuclei such as nucleus raphe pallidus, nucleus raphe magnus, or nucleus raphe obscurus (data not shown).

DISCUSSION

The findings of the present investigation demonstrate for the first time, using an anterograde tracing technique, that projections from caudal PH and MVePC terminate within the DRN in rats. Previous electrophysiological and retrograde tracing studies have provided evidence that the DRN is targeted by projections from the vestibular nuclei and PH. For example, Kalén et al. [13] observed small numbers of retrogradely-labeled cells in SVN after injection of wheat germ agglutinin-horseradish peroxidase (WGA-HRP) into the DRN, and Lee et al. [17] reported that injection of the WGA-HRP derivative WGA-apo-HRP-gold into DRN produces retrograde labeling in PH. It has been reported [14] that injections of horseradish peroxidase (HRP) into the caudal DRN in cats produced retrogradely-labeled cells within MVN and SVN; these workers were also reportedly able to orthodromically activate neurons in caudal DRN by electrically stimulating either MVN or SVN. However, based on the described location of their sites of HRP injection and electrophysiological recording, it is doubtful that these recordings and tracer injections were actually located within the DRN. Likewise, Sakai et al. [29] observed retrograde labeling in PH, SVN, and MVN after HRP injection into the DRN of cats, but only from those injection cases that also involved the mlf. The present findings confirm that the DRN is targeted by projections from caudal MVePC and PH, and demonstrates that those projections ascend to DRN through the mlf fiber bundle. The MVePC and PH send projections most extensively to the lateral part of DRNvm and the ventral part of DRNI, although the PH also projected to a wide region of the DRN. This finding suggests that input from the vestibular nuclear complex is likely to heavily influence the activity of neurons located within ventrolateral DRN.

Extensive evidence demonstrates that the different subdivisions of the DRN receive distinct types of afferent input [25,26], display dissimilar projection patterns [22,34,37,39,40], and are neurochemically unique [23,31]. Studies have shown that the midline DRN sends projections to both cortical and subcortical sites, whereas the lateral aspect of the DRN projects primarily to subcortical regions [22,34,37,39,40]. An anterograde tracing study of DRN projections [7] has demonstrated that the vestibular nuclei are heavily innervated by projections from the lateral and ventromedial subdivisions of the DRN. The present investigation demonstrates that

two regions of the vestibular nuclear complex [28]—caudal MVePC and PH—are sources of projections to DRNI and DRNvm. Thus, the subdivisions of the DRN that project heavily to the vestibular nuclei are also selectively targeted by projections from the vestibular nuclei.

The DRN contains both serotonergic and nonserotonergic neurons [20]. Waterhouse and colleagues [41] found that serotonergic DRN cells in behaving rats are generally insensitive to sensorimotor stimulation whereas nonserotonergic DRN neurons display responses to specific sensory and motor events. However, there is evidence that a subpopulation of serotonergic DRN neurons increase their firing during motor activities such as grooming, chewing, and walking, but decrease their activity during orienting responses [11,12]. Based on these findings, Jacobs and Fornal [12] have proposed that one function of serotonergic DRN neurons is to simultaneously facilitate motor output and inhibit sensory information processing during execution of motor programs. Cells in MVePC and PH show eye movement-related firing activity [1,2,4,15,27], and it is possible that input from MVePC and PH may serve to inhibit the firing of serotonergic DRN neurons during eye movement. The DRN contains a substantial number of GABAergic neurons [5,21,32], and these cells are a major source of inhibitory input to serotonergic DRN neurons [18,38]. It is conceivable that GABAergic DRN interneurons are targeted by projections from MVePC and PH. Activation of GABAergic interneurons by input from MVePC and PH would induce feed-forward inhibition of serotonergic DRN neurons, potentially contributing to the inhibition of serotonergic cell firing that occurs during orienting behaviors. Nonetheless, regardless of the exact influence of MVePC and PH input on DRN activity, the present findings confirm that these regions of the vestibular nuclear complex project to discrete regions of DRN.

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Figure 1.

Photomicrographs of BDA injection sites. (A) BDA injection site located in the medial part of the parvicellular division of medial vestibular nucleus (MVePC) of case 1. (B) BDA injection site located within the ventrolateral part of the rostral nucleus prepositus hypoglossi (PH) of case 25. Abbreviations: g7, genu of the facial nerve; MVePC, medial vestibular nucleus, parvicellular division. Scale bars = 200 μ m.

Figure 2.

Distribution of anterograde labeling in the dorsal raphe nucleus (DRN) after injection of BDA into (A) the medial part of the parvicellular division of medial vestibular nucleus (MVePC) in animal 1, and (B) the ventromedial part of caudal prepositus hypoglossi (PH) with some spread into the mlf in animal 26. For both injection cases, BDA-labeled fibers are charted in camera lucida drawings of two transverse sections located at the indicated AP levels (relative to bregma). Transport ipsilateral to the injection site is illustrated on the right side of the figure. Abbreviations: DRNm, medial division of the dorsal raphe nucleus; DRNI, lateral division of the dorsal raphe nucleus; mlf, medial longitudinal fasciculus; IV, trochlear nucleus.

Figure 3.

Photomicrographs of BDA-labeled fibers in the DRN. (A) Anterograde labeling in the ventromedial DRN after injection of BDA into the parvicellular division of medial vestibular nucleus. (B) Anterograde labeling in the dorsal aspect of lateral DRN after injection of BDA into nucleus prepositus hypoglossi. (C, D) BDA-labeled terminals (arrows) interspersed among 5-HT-positive neurons (indicated by asterisks) in the dorsomedial (C) and lateral (D) subdivisions of the DRN. Scale bars = 50 μ m in A, C and D, and 20 μ m in B.