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Projections from the Spinal Trigeminal Nucleus to the Cochlear Nucleus in the Rat

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ABSTRACT

The integration of information across sensory modalities enables sound to be processed in the context of position, movement, and object identity. Inputs to the granule cell domain (GCD) of the cochlear nucleus have been shown to arise from somatosensory brain stem structures, but the nature of the projection from the spinal trigeminal nucleus is unknown. In the present study, we labeled spinal trigeminal neurons projecting to the cochlear nucleus using the retrograde tracer, Fast Blue, and mapped their distribution. In a second set of experiments, we injected the anterograde tracer biotinylated dextran amine into the spinal trigeminal nucleus and studied the resulting anterograde projections with light and electron microscopy. Spinal trigeminal neurons were distributed primarily in *pars caudalis* and *interpolaris* and provided inputs to the cochlear nucleus. Their axons gave rise to small (1–3 μm in diameter) en passant swellings and terminal boutons in the GCD and deep layers of the dorsal cochlear nucleus. Less frequently, larger (3–15 μm in diameter) lobulated endings known as mossy fibers were distributed within the GCD. Ventrally placed injections had an additional projection into the anteroventral cochlear nucleus, whereas dorsally placed injections had an additional projection into the posteroventral cochlear nucleus. All endings were filled with round synaptic vesicles and formed asymmetric specializations with postsynaptic targets, implying that they are excitatory in nature. The postsynaptic targets of these terminals included dendrites of granule cells. These projections provide a structural substrate for somatosensory information to influence auditory processing at the earliest level of the central auditory pathways. J. Comp. Neurol. 484:191–205, 2005.

Indexing terms: granule cells; mossy fibers; polysensory convergence; synapses

Hearing involves more than processing information about vibrations in air. As an animal moves its head or pinna, the acoustic cues used to locate sounds in the environment are changing. Knowledge of head and pinna position is necessary for the auditory system to interpret these changes. Vestibular and visual information can be used to determine relative motion between the organism and a sound source. Proprioceptive input might help the auditory system to distinguish between environmental and self-generated sounds (e.g., vocalizations, chewing). Identifying a sound also requires learning and memory. That is, sounds made by conspecifics will differ from those of a predator, and they will evoke different affective states. In this context, we seek pathways in the brain that might integrate these various functions.

The substrate for some of this integration is evident in the cochlear nucleus. The cochlear nucleus receives input from a variety of nonauditory structures. The sources include the dorsal column nuclei (Itoh et al., 1987; Wein-

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berg and Rustioni, 1987; Wright and Ryugo, 1996; Li and Mizuno, 1997a,b), Scarpa's ganglion and the medial vestibular nucleus (Burian and Gstoettner, 1988; Kevetter and Perachio, 1989; Bukowska, 2002), the trigeminal ganglion (Shore et al., 2000) and nuclei (Itoh et al., 1987; Wright and Ryugo, 1996; Li and Mizuno, 1997a,b; Wolff and Künzle, 1997), and the pontine nuclei (Ohlrogge et al., 2001). These projections emphasize the idea that the meaning of a sound depends on more than its physical characteristics.

In the dorsal cochlear nucleus (DCN) of the cat, the response properties of projection neurons (e.g., pyramidal and giant cells) are modulated by manipulating the pinna or electrically stimulating the nerve carrying proprioceptive afferents from areas that include the pinna (Saadé et al., 1989; Young et al., 1995; Kanold and Young, 2001). These results indicate that somatosensory activity influences auditory neurons but it is not entirely known which circuits of the somatosensory system convey the information. Some neurons in the primary relay nuclei of the somatosensory system send their axons into the cochlear nucleus (e.g., cat, Itoh et al., 1987; rat, Weinberg and Rustioni, 1987; Wright and Ryugo, 1996; Li and Mizuno, 1997; hedgehog, Wolff and Künzle, 1997) but there are differences in the details of the observations. In the cat, brainstem somatosensory projections were restricted exclusively to the granule cell domain (GCD, Itoh et al., 1987), whereas the projection in the rat was "almost completely confined" to the DCN, to the superficial granular layer of the ventral cochlear nucleus, and to the subpeduncular corner of granule cells (Weinberg and Rustioni, 1987). In contrast, more recent reports in rat and hedgehog report somatosensory projections throughout the GCD and the deep layers of the DCN (Wright and Ryugo, 1996; Wolff and Künzle, 1997). The projection in the hedgehog, however, is sparse compared to that observed in the rat. Curiously, light touch to the head and neck and movement of the vibrissae, all mediated through the trigeminal nerve, were ineffective in evoking responses in DCN neurons in the cat (Kanold and Young, 2001). These variations across species and/or methods merit further investigation if we are to understand the mechanisms of polysensory integration.

Resolving these details is important because trigeminal terminals in the central core of the cochlear nucleus imply a direct influence on the projection cells giving rise to the ascending auditory pathways. In contrast, trigeminal input confined to the GCD suggests an indirect effect on auditory processing because the GCD is composed of interneurons (Mugnaini et al., 1980a; Wright and Ryugo, 1996). We were also interested in the synaptic terminals formed by this pathway in the GCD. The GCD contains a particular type of synaptic ending called a mossy fiber (McDonald and Rasmussen, 1971; Mugnaini et al., 1980b; Wright and Ryugo, 1996; Weedman et al., 1996). Mossy fibers are distinguished by their large size, irregular shapes, and numerous synapses formed with microneurons in the GCD (Mugnaini et al., 1980b; Hutson and Morest, 1996; Weedman et al., 1996; Weedman and Ryugo, 1996). Mossy fiber endings arise from the cuneate nucleus (Wright and Ryugo, 1996) and pontine nuclei (Ohlrogge et al., 2001) but these sources account for only a small proportion of the mossy fibers in the GCD.

Somatosensory inputs are hypothesized to provide information regarding pinna orientation to aid in the analysis of sound localization cues (Young et al., 1995; Kanold and Young, 2001). To what extent the trigeminal complex fits into this scheme is unknown. The goal of this study was to describe the source and target(s) of the trigeminal pathway to the CN. Our working hypothesis is that the identification of these cells and circuits will help us understand the role of each pathway for sound perception.

We used retrograde tracing methods to map the distribution of cells in the spinal trigeminal nucleus that project to the CN. We found that the predominant source for this pathway originates in the caudal divisions of the nucleus. In a second set of experiments, we injected anterograde tracers in these caudal regions and examined the distribution and type of labeled terminals produced in the CN. Terminals were primarily observed in the GCD but many were also located in the central core of the DCN and the ventral CN (VCN). The ultrastructural features of some large terminals in the GCD defined them as mossy fibers. They resemble those that arise from the cuneate nucleus and appear to target the dendritic claws of granule cells.

MATERIALS AND METHODS

Animals and animal preparation

A total of 21 male Sprague-Dawley rats, weighing 250–350 g, were used in this study. Animals were deeply anesthetized with an intraperitoneal injection of sodium pentobarbital (45 mg/kg) and given an intramuscular injection of 0.1 cc of atropine to reduce secretions. Lidocaine was infiltrated around the ear canals and the surgical areas. When the animal was areflexic to a paw pinch and had no corneal reflex, surgery was initiated. All procedures were in accordance with established NIH guidelines and approved by the Animal Care and Use Committee of the Johns Hopkins School of Medicine.

Retrograde labeling. We used retrograde tracers to label cells in the trigeminal nuclei that project to the CN in the rat. The source of this pathway is located primarily in the caudal regions of the spinal trigeminal nuclei (Itoh et al., 1987; Wolff and Künzle, 1997). However, neurons in rostral portions of these nuclei (e.g., *pars oralis*) project to the CN in the hedgehog *tenrec* (Wolff and Künzle, 1997), whereas they may not in the cat (Itoh et al., 1987). Our goal was to examine this issue in the rat and also provide a target for the forthcoming anterograde studies. In these retrograde experiments, tracer was injected in the granule cell lamina (GCL) situated between the DCN and VCN because the GCD receives most of the input from trigeminal cells (Itoh et al., 1987; Wolff and Künzle, 1997).

A skin incision was performed to expose the occipital cranium. The posterior calvarium was drilled away in order to expose the underlying cerebellum. The dura was cut and cerebellar tissue was aspirated in order to reveal the dorsal and lateral aspects of the left cochlear nucleus. Using an operating microscope, a glass micropipette (40 μm tip ID) filled with a 3% aqueous solution of Fast Blue (FB, Sigma Chemical, St. Louis, MO) or 10% biotinylated dextran-amine (BDA, mw 10,000, Molecular Probes, Eugene, OR) was aimed at the GCL and inserted 300–500 μm below the dorsolateral surface of the CN. A pressure injector (Drummond Scientific, Broomall, PA) was used to inject 27.6 nl of the FB solution (n = 10). BDA was injected using positive current pulses (5 μA, 7 seconds on, 7 seconds off) for 5–10 minutes (n = 3). Gelfoam was placed

over the aspirated surface of the cerebellum, the skin incision was sutured, and the animal was allowed to recover. After 4–6 days, the rats were anesthetized with an overdose of sodium pentobarbital (100 mg/kg) before being transcardially perfused with 10% formalin in 0.1 M phosphate buffer (PB; pH 7.4).

Anterograde labeling. Rats (n = 8) were anesthetized with an intraperitoneal injection of sodium pentobarbital solution and held in a stereotaxic frame. Soft tissue overlying the occipital bone was removed and the posterior aspect of the left cerebellar cortex exposed by drilling. The dura was incised and the lateral and floccular cerebellum partially aspirated until achieving satisfactory exposure of the underlying brain stem. The anterograde tracer, 10% (w/v) BDA, was stereotaxically injected into the spinal trigeminal nucleus (Sp5). Injections were made in the *pars interpolaris* and *pars caudalis* divisions of the nucleus because the retrograde labeling experiments showed that most cells projecting to the CN were located in these regions. Tracer solution (27.6 nl) was injected using a glass pipette (20–30 μm I.D.) coupled to a pressure injector (Drummond Scientific). Animals were sutured and allowed to recover for 7–10 days before being anesthetized with an overdose of sodium pentobarbital and transcardially perfused with 4% paraformaldehyde in 0.1 M PB (pH 7.4).

Tissue processing and analysis of retrograde data

Brains and trigeminal ganglia from rats with CN injections were dissected from the skull, postfixed for 1 hour at room temperature, and embedded in blocks of gelatin-albumin hardened with 4% paraformaldehyde. Coronal slices (50 μm thick) of the brain, from cervical spinal cord to olfactory bulbs, were made using a Vibratome. The sections were collected in serial order in PB (pH 7.4) and alternate sections were mounted on microscope slides. Every section from the trigeminal ganglia was collected and mounted on slides. Slides were then air-dried and coverslipped (Krystalon, EM Science, Gibbstown, NJ) prior to examination with a fluorescent microscope (Zeiss Axioplan). The alternate series of sections was stained with cresyl violet and used to identify the location of retrogradely labeled cells with the aid of a published map of brain nuclei for the rat (Paxinos and Watson, 1998). BDA tissue was processed as described below for anterograde labeling.

The distribution of FB-labeled cells throughout the brain was documented in the following way. Each cresyl violet-stained section was matched with the corresponding atlas section (Paxinos and Watson, 1998) using section shape and nuclear landmarks. We plotted the position of labeled cells in each fluorescent section for all experimental cases using a total magnification of ×200. Each section was scaled and aligned to best match an atlas section (PhotoShop v. 6.0, Adobe, San Jose, CA). In some cases, labeling patterns or individual cells were photographed with a CCD camera (Hamamatsu C5810) connected to a Macintosh G4 computer.

Tissue processing and analysis of anterograde data

The brain was dissected from the skull and the brain stem separated from the spinal cord, cerebellum, and forebrain. Brain stems were postfixed for 1 hour at room temperature and embedded in blocks of gelatin-albumin hardened with 4% paraformaldehyde. Coronal sections (50 μm thick) were cut using a Vibratome, spanning the cervical spinal cord through the cochlear nucleus, and collected in serial order in PB. Alternate sections were incubated overnight with ABC Elite solution (Vector Laboratories, Burlingame, CA) at 5°C and reacted with diaminobenzidine (DAB) using standard techniques to reveal reaction product. Every third section through the cochlear nucleus in this batch was then immediately prepared for electron microscopic processing. After incubation in 1% osmium tetroxide for 15 minutes, these sections were washed, stained en bloc with 1% uranyl acetate overnight, dehydrated, infiltrated with Epon, and embedded between sheets of Aclar. Sections were hardened overnight at 70°C and taped to microscope slides for light microscopic analysis. The unprocessed sections were mounted on chrom-alum-treated microscope slides and air-dried. These sections were counterstained with cresyl violet and coverslipped with Permount. The cochlear nucleus was drawn at low magnification (×125) with the aid of a light microscope and drawing tube using blood vessels, tissue tears, and nuclear borders as landmarks. Labeled swellings were then plotted onto these drawings using a 40× objective (total magnification ×500) guided by the tissue landmarks.

Ultrastructure

Relevant sections were photographed at a total magnification of ×25 and ×100. The region of the cochlear nucleus containing labeled cells was cut from the Aclar and reembedded in Epon in BEEM capsules. Capsules were hardened at 70°C and block faces trimmed for ultrathin sectioning. The position of labeled endings relative to blood vessels, tissue borders, and neighboring cells was mapped using a light microscope and drawing tube (×125 and ×250 magnification). A semithin section (250 nm) was cut and stained with Toluidine blue for orientation in the electron microscope. Serial ultrathin sections (75 nm) were taken through selected labeled endings. Sections were collected on slotted grids coated with Formvar, counterstained with 7% uranyl acetate, and maintained in serial order. Ultrathin sections were viewed and photographed with a JEOL 100CX electron microscope.

RESULTS

Retrograde labeling in the spinal trigeminal nucleus

Results presented here are derived from 13 rats with injections into the CN. The injection sites were centered in or near the GCL (Figs. 1, 2). FB and BDA injections tended to spread outside the GCL and into the lateral regions of the DCN and/or the dorsal regions of the PVCN. In no case, however, was tracer found rostral to the PVCN. The injections were necessarily near cerebellar fiber tracts such as the inferior cerebellar peduncle, raising the possibility of retrograde labeling produced by "axons of passage." For this report, however, the injection sites were unambiguously confined to the CN (e.g., Figs. 1A, 2). The halo of the dye remained within the nucleus, although small flecks of fluorescence were scattered about on a few occasions. These flecks were found within a few millimeters of the injection site and were not contained within

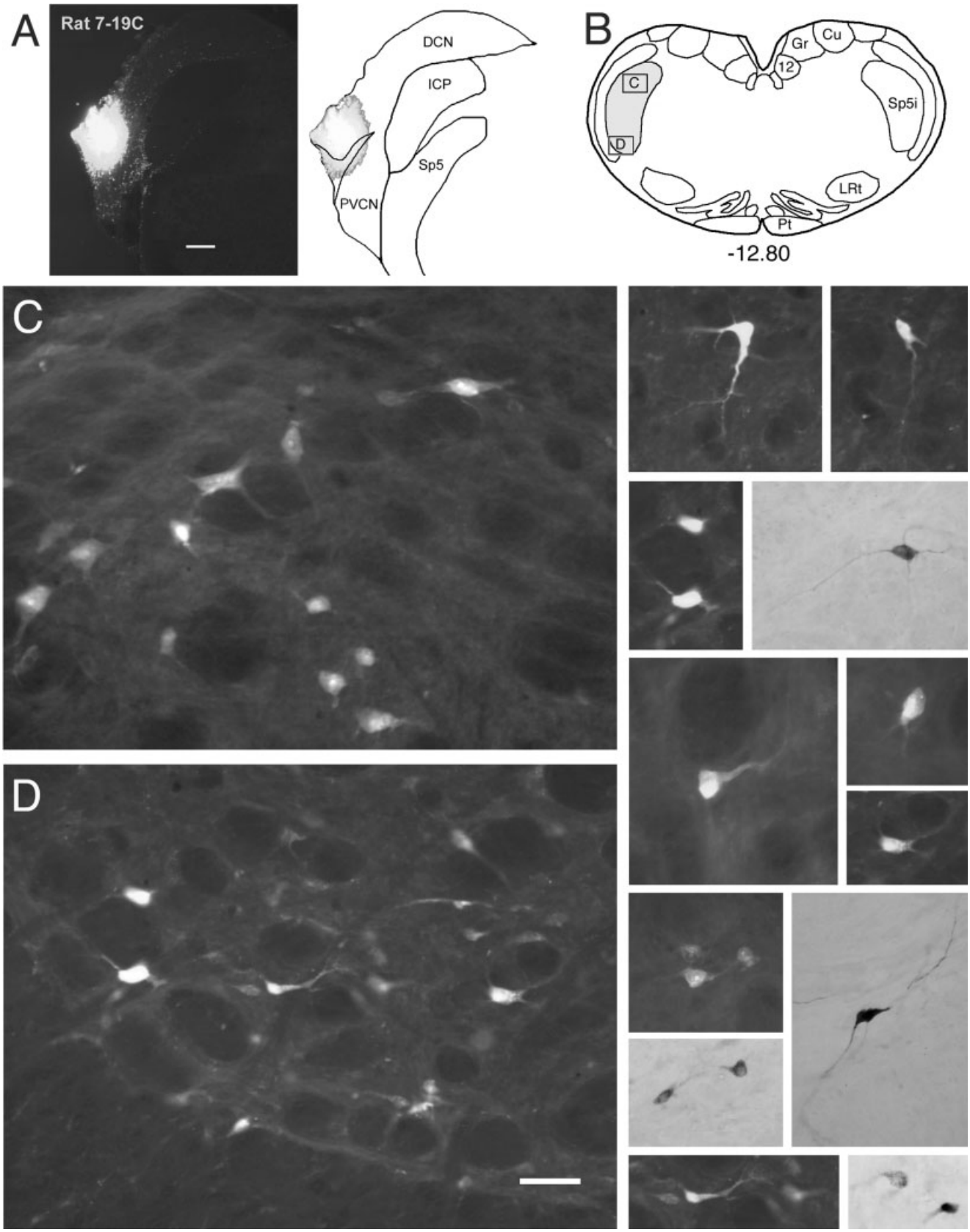

Fig. 1. Photomicrographs of retrogradely labeled neurons in the spinal trigeminal nucleus. **A:** Photomicrograph and drawing of coronal sections through the cochlear nucleus showing the circumscribed injection site for Fast Blue. The injection was entirely confined within the nucleus and encroached upon the granule cell domain. The lack of fluorescence on the tissue surface is evidence that tracer did not leak out of the injection site and contaminate other structures. **B:** Drawing of coronal section through the brain stem and spinal trigeminal nucleus indicating the locations of photomicrographs in the dorsal (**C**) and ventral (**D**) region of *pars interpolaris*. The remaining cells (right panel) were distributed throughout the nucleus. These multipolar cells were labeled by Fast Blue or BDA, and their cell bodies range from 18–40 μm in long axis length. 12, hypoglossal nucleus; Cu, cuneate nucleus; Gr, gracile nucleus; LRt, lateral reticular nucleus; Pt, pyramidal tract; Sp5i, spinal trigeminal nucleus, *pars interpolaris*. Scale bars = 300 μm in A; 50 μm in D (applies to photomicrographs of all labeled cells).

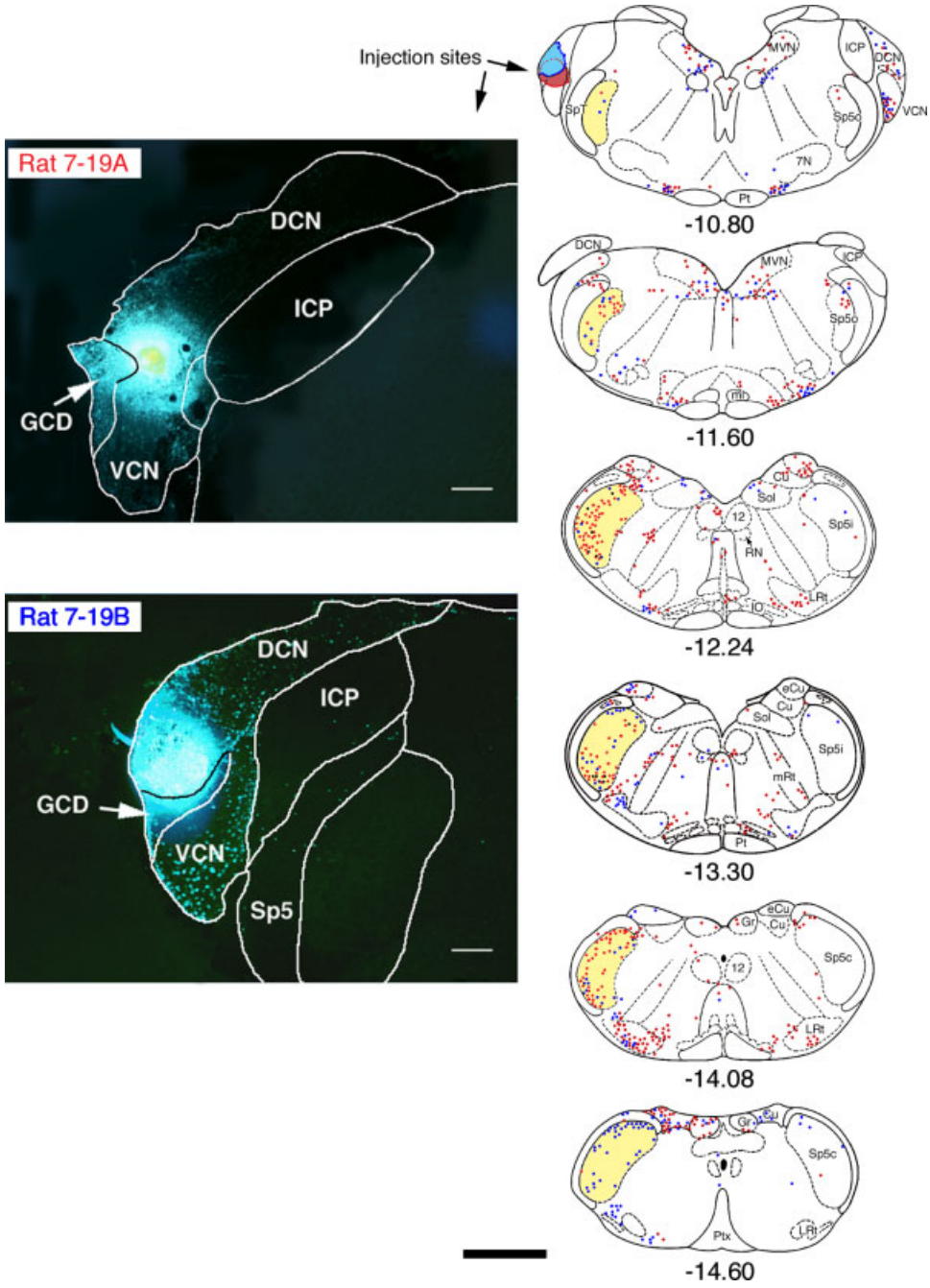

Fig. 2. Following unilateral Fast Blue injections in the cochlear nucleus, labeled cells were distributed throughout the neuraxis. Such labeled cells are represented as dots on atlas sections of the rat brain stem (Paxinos and Watson, 1998). Section location is indicated with respect to distance from bregma, proceeding from rostral to caudal. Representative data from two rats are illustrated (Rat 7-19A, red symbols; Rat 7-19B, blue symbols). Note especially the labeling found in the spinal trigeminal nucleus, *pars caudalis* and *pars interpolaris*. 7N, facial motor nucleus; 12, hypoglossal nucleus; Cu, cuneate nucleus; DCN, dorsal cochlear nucleus; eCu, external cuneate nucleus; Gr, gracile nucleus; ICP, inferior cerebellar peduncle; IO, inferior olive; LRt, lateral reticular nucleus; ml, medial lemniscus; mRt, medullary reticular nuclei; MVN, medial vestibular nucleus; Pt, pyramidal tract; Ptx, pyramidal decussation; RN, Roller's nucleus; Sol, nucleus and tract of the solitarius; Sp5c, spinal trigeminal nucleus, *pars caudalis;* Sp5i, spinal trigeminal nucleus, *pars interpolaris*; SpCo, spinal trigeminal nucleus, *pars oralis*; Scale bars = 300 μm in left panels; 2 mm for atlas sections.

cells (Rat 7–19B, Fig. 2). The results described below represent observations common to all animals.

In each case, we observed retrogradely labeled cells in the spinal trigeminal nuclei. These neurons were heterogeneous with respect to size and shape, consisting primarily of large (>25 μm in diameter) and small (<20 μm in diameter) multipolar cells (Fig. 1). Small and large cells were intermingled (Fig. 1C,D) and we did not observe any differences in their distribution. The location of such labeled cells is plotted for two representative cases (Fig. 2). There were few labeled cells found in the main sensory nucleus, and no labeled cells were found in the mesencephalic or motor nucleus. Data from the separate animals are distinguished using different colors (red and blue). The majority of labeled cells were observed ipsilateral to the injection site. Labeled cells were found along the entire rostral-caudal length of the spinal trigeminal nucleus. A few were sprinkled in cell clusters embedded within the descending tract of the trigeminal nerve called interstitial neurons (Ramón y Cajal, 1909). There was preferential labeling in the caudal part of *pars interpolaris* (Fig. 2, sections –12.24 and –13.30) and throughout *pars caudalis* (Fig. 2, sections –14.08 and –14.60). In most animals (e.g., Rats 7–19A and 7–19B in Fig. 2), the labeled cells tended

to be distributed towards the lateral side of the *pars caudalis*. In *pars interpolaris*, the distribution was biased toward the ventral half of the nucleus.

It should be mentioned that retrogradely labeled neurons with ascending axons were also found in the dorsal column nuclei including the external cuneate nucleus, the medial vestibular nuclei, the medullary reticular nuclei, the hypoglossal nucleus, and Roller's nucleus (Fig. 2). Neurons with descending axons were found in the superior olivary complex, nuclei of the lateral lemniscus, locus coeruleus, pontine nuclei, inferior colliculus, deep layers of the superior colliculus, nucleus of the dorsal raphé, and auditory cortex (data not shown). Commissural projections arose from the contralateral cochlear nucleus. Projections from many of these structures have been reported previously (Covey et al., 1984; Itoh et al., 1987; Spangler et al., 1987; Weinberg and Rustioni, 1987; Spirou and Berrebi, 1996; Ohlrogge et al., 2001; Schofield, 2001), whereas others need further study because of the possible involvement of the injection site with the cerebellum. The present report is focused on the spinal trigeminal nucleus.

We explicitly dissected and studied the trigeminal ganglion both ipsilateral and contralateral to the injection site. Evidence has been presented indicating that these ganglion cells project directly to the GCD and the central core of the VCN in guinea pigs (Shore et al., 2000, 2003). We observed only a single labeled ganglion cell in one rat. Evidence for a direct projection from trigeminal afferents to the CN was not present in our data but it should be emphasized that our injections were small and occupied only a small portion of the GCD and usually the DCN.

Anterograde labeling in the cochlear nucleus

In eight rats, BDA injection sites were centered in either *pars interpolaris* (Figs. 5, 6; n = 5) or *pars caudalis* (Fig. 3; n = 3). BDA reaction product was confined to a single subdivision. Injections were observed lateral and ventral to the dorsal column nuclei (cuneate and gracile nuclei), a known source of projections to the CN. A few labeled neurons (range, 5–20) were sprinkled rostral and caudal to the injection site within the spinal trigeminal nucleus. There were no retrogradely labeled cells or anterogradely labeled fibers and terminals detected in the dorsal column nuclei. Ascending projections to the thalamus exhibited a dense focus of labeling in the medial division of the contralateral ventral posterior nucleus, the accepted target of trigeminal projections (Veinante et al., 2000). There were only a few terminals in the lateral division of the ventral posterior nucleus, the accepted target of dorsal column neurons (data not shown). These results were consistent with reported thalamic projection patterns from the somatosensory brainstem nuclei (Peschanski, 1984).

Light microscopic observations

Labeled axons emerged from the injection site and generated collaterals within the spinal trigeminal nucleus. Other collaterals were observed to arch laterally over, under, or directly through the spinal tract of the trigeminal nucleus and make their way towards the ipsilateral CN. Fibers entered the ipsilateral CN through the ventral, intermediate, or dorsal acoustic stria. Fibers projecting to the contralateral side passed briefly in the ipsilateral midbrain tract of the trigeminal nerve and continued medially into the medial longitudinal fasciculus (MLF). Fibers crossed the midline in the MLF, continued laterally as they traversed the MLF and the contralateral midbrain tract of the trigeminal nerve, and entered the contralateral CN through the dorsal acoustic stria. In the VCN, fibers tended to run within or near the GCD as they gave off terminals. Within the DCN, fibers ran laterally within the underlying white matter and then angled up into the deep layer while maintaining a lateral trajectory. In the magnocellular core of the CN, fibers were less frequent and exhibited random orientations.

Three types of axonal swellings were observed in the CN (Fig. 3). Small (1–3 μm in diameter) en passant swellings were distributed along thin stretches of the axons. These swellings resembled "beads on a string" and were found primarily on the terminal branches of the projecting arborizations. Similarly small (1–3 μm in diameter) terminal swellings were found at the tips of collateral branches. Both kinds of small swellings were found in close proximity to each other and shared a common distribution pattern throughout the GCD and the central core of the CN. The third type of swelling had a wide range of sizes (3–15 μm in diameter) and many could be quite large (Fig. 4). Besides their size, they were distinguished by their irregular, lobulated shape and finger-like projections that sprouted from the body of the swelling (Fig. 4, far left panel). These distinctive swellings are referred to as mossy fibers because of their structural similarity to mossy fibers of the cerebellum. Mossy fibers were restricted to the GCD and they were far outnumbered by the smaller terminal swellings.

Labeled swellings were concentrated in the GCD, the deep DCN, and to a lesser extent, in the magnocellular core of the VCN (Figs. 5, 6). The vast majority of the swellings were observed in the CN ipsilateral to the injection site. These patterns did not depend on the rostral/caudal location of the injection. There was, however, a difference in the pattern within the VCN as a function of the dorsal/ventral location of the injection site. Injections located in the ventral half of the spinal trigeminal nucleus (*pars interpolaris*, n = 2; *pars caudalis*, n = 1) produced an additional projection to the AVCN (Fig. 5, sections 1, 4, 10). In contrast, injections centered in the dorsal half of the nucleus (*pars interpolaris*, n = 2) yielded labeled terminals in the PVCN (Fig. 6, sections 20, 22, 24). The remaining three cases had injection sites that spanned the dorsoventral extent of the spinal trigeminal nucleus and swellings were observed in all magnocellular divisions of the CN.

Electron microscopic observations

Selected examples of en passant swellings, terminal boutons, and mossy fibers were examined with an electron microscope. All contained features indicative of synapses. These features consisted of a punctate membrane thickening and the presence of round synaptic vesicles (Fig. 7). Synaptic vesicles within the labeled ending were present around the membrane thickening. The facing membrane of the adjacent profile was also marked by a thickening. The paired membrane thickenings tended to be similar in length but not in thickness; consequently, the complex exhibited an asymmetric appearance. The postsynaptic membrane was usually uniformly thick and fuzzy, whereas the presynaptic membrane was marked by one or

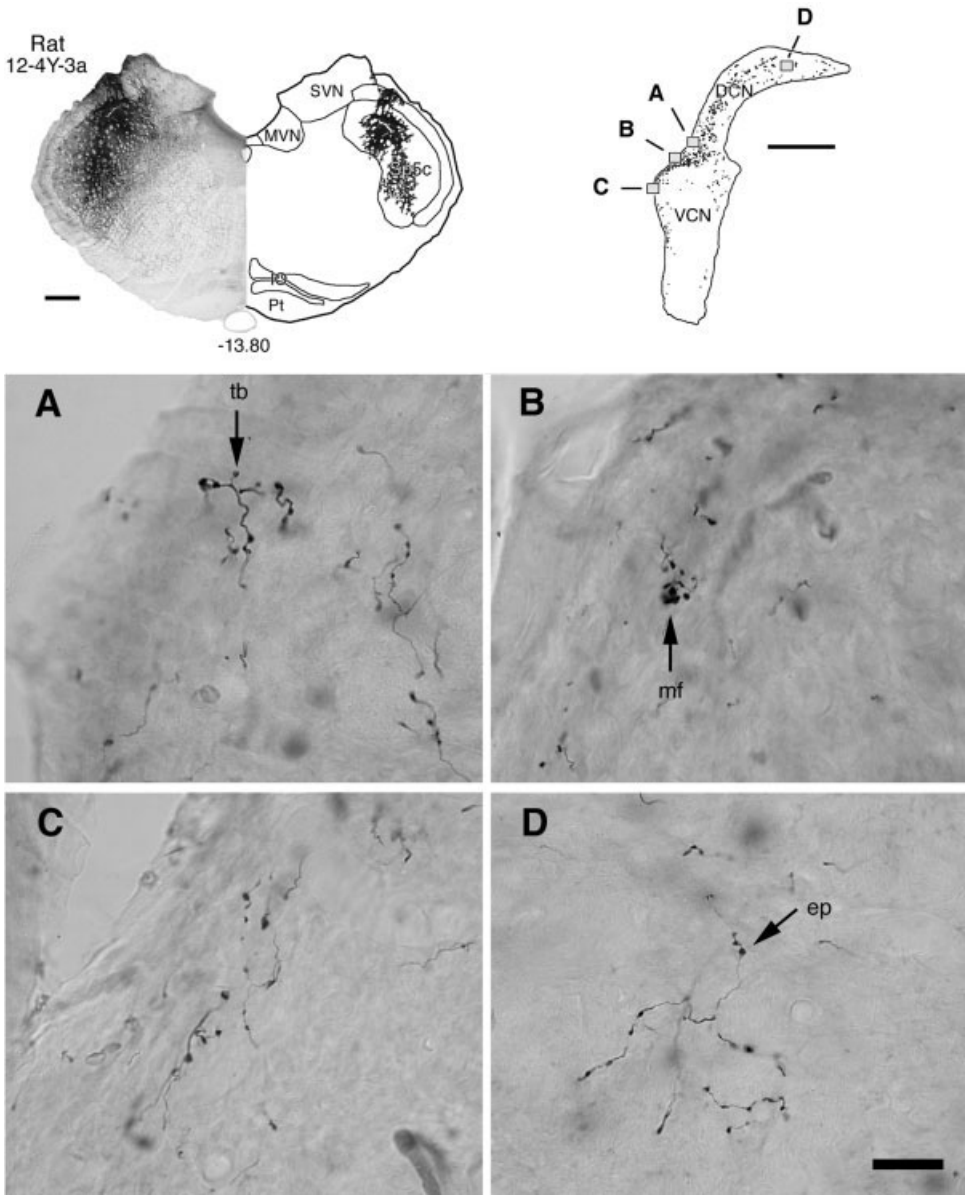

Fig. 3. Photomicrographs illustrate anterogradely labeled fibers and endings in the cochlear nucleus that arise from the ipsilateral spinal trigeminal nucleus. Top left panel: Photomicrograph and drawing of a coronal section through the brain stem, illustrating the injection site in the spinal trigeminal nucleus, *pars caudalis*. Top right panel: Drawing of a coronal section through the cochlear nucleus illustrating the locations of the photomicrographs (A–D). Dots on the drawing indicate individual endings. **A:** Fibers and endings in the dorsolateral part of the granule cell lamina. Arrow indicates terminal bouton (tb). **B:** Large ending near the center of the micrograph is a mossy fiber (mf). This region is along the lateral edge of the granule cell lamina. **C:** Fibers and endings along the superficial lamina on the lateral edge of the VCN. **D:** This spray of endings is in the middle of the deep layer of the DCN. Arrow indicates an en passant swelling (ep). Scale bars = 0.5 mm for top panels; 20 μm in D (applies to A–D).

several dense projections. Synapses were formed on smooth, dendritic profiles. These postsynaptic dendritic profiles exhibited mitochondria and microtubules that are typical of granule cell dendrites (Mugnaini et al., 1980b; Weedman and Ryugo, 1996). Other bouton terminals in the GCD and the magnocellular core of the CN formed synapses with the dendritic shafts of unidentified neurons.

The most prominent synaptic endings were mossy fibers located exclusively in the GCD. When viewed with the electron microscope, mossy fibers were irregular in shape and harbored numerous mitochondria that were centrally localized (Figs. 7, 8). Mossy fibers generated by trigeminal axons were identifiable by the presence of HRP-DAB reaction product that darkened the cytoplasm. This reaction product also adhered to the outer surface of the synaptic vesicle membrane forming a dark halo and emphasizing the round, pale lumen. The perimeter of the mossy fiber was incompletely studded with dendritic profiles and the entire complex was loosely ensheathed by several layers of glial lamellae (Fig. 7). Fine digitiform appendages and hair-like extensions arose from the surrounding dendrites and penetrated into the mossy fiber. Granule cells generate 2–4 primary dendrites, each of which terminates in a tuft of branches that has a claw-like appearance (Mugnaini et al., 1980a; Weedman et al., 1996; Doucet and Ryugo, 1997). These features match those of the synaptic complexes formed by trigeminal mossy fibers (Fig. 8) and thus we infer that the postsynaptic dendrites arise from granule cells.

Labeled mossy fibers were found among unlabeled mossy fibers. The neuropil of the GCD is characterized by many kinds of endings, and even with a large projection into the GCD from the spinal trigeminal nucleus, the vast

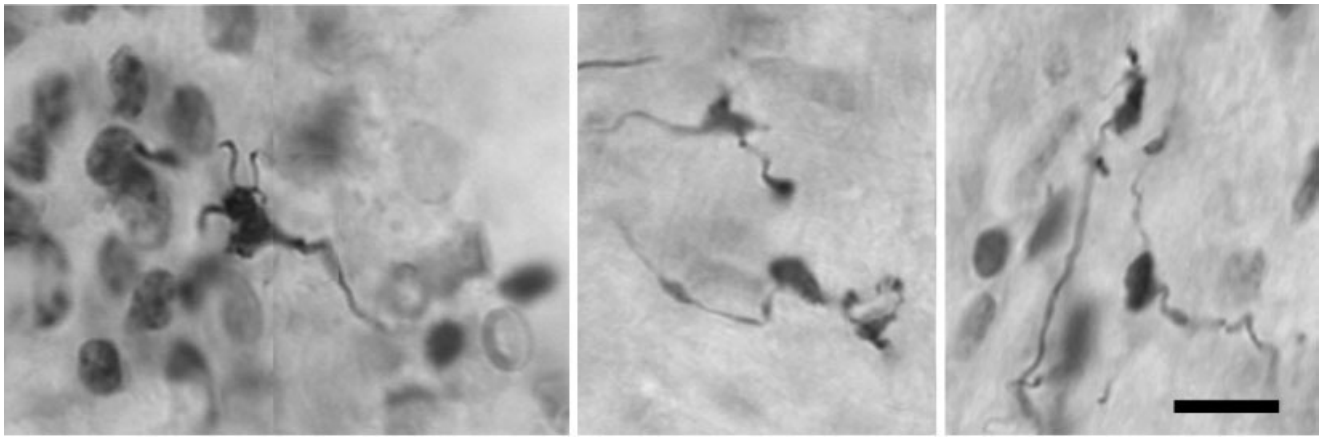

Fig. 4. Photomicrographs illustrate labeled mossy fibers. Mossy fibers are distinguished from terminal boutons by their larger size and lobulated appearance. Filiform appendages that terminate in small swellings are also evident. Mossy fibers of the cochlear nucleus are not nearly as large or complicated in shape as those found in the cerebellar cortex (Palay and Chan-Palay, 1974), but they are distinctive nonetheless. In the cochlear nucleus, mossy fibers from the spinal trigeminal nucleus are found entirely within the GCD. Scale bar = 10 μm.

majority of endings are unlabeled. Resident neurons consisted of granule cells, Golgi cells, unipolar brush cells (UBCs), and chestnut cells (Mugnaini et al., 1980b, 1997; Weedman et al., 1996; Doucet and Ryugo, 1997; Spatz, 2000; Alibardi, 2003). Mitt cells, described by Morest and his colleagues in the chinchilla (Hutson et al., 1999; Hutson and Morest, 1996), resemble UBCs but they differ because where mitt cells receive inputs to their dendritic stalk, UBCs do not. It remains to be determined if the characteristics of mitt cells and UBCs reflect species differences, or true variations in cell types. We could not identify every structure postsynaptic to a labeled ending, but those that we could identify were always granule cells or their dendrites.

DISCUSSION

Spinal trigeminal projections

The present study concentrates on projections from the spinal trigeminal nucleus to the ipsilateral cochlear nucleus. There is a minor projection to the contralateral cochlear nucleus. This spinal trigeminal-cochlear nucleus pathway arises primarily from the middle and caudal regions of the nucleus. The axons ascend in the spinal tract of the trigeminal nerve to enter the cochlear nucleus. They projected throughout the GCD and the deep layers of the DCN, branched, and gave rise to distinct terminal endings, and exhibited asymmetric membrane specializations and round synaptic vesicles.

The projection from the spinal trigeminal nucleus to the cochlear nucleus is not uniform. The dorsal region of the spinal trigeminal nucleus not only projects to the GCD and deep DCN but has an additional projection to the PVCN. In contrast, the ventral region exhibits an extra projection to the AVCN. These patterns held for both *pars interpolaris* and *caudalis*. Furthermore, injections of BDA in one part of the nucleus labeled fibers and a few dozen somata along the rostral-caudal extent of the ipsilateral nucleus. This result implies that individual neurons tend to give rise to collaterals that distribute within the spinal trigeminal nucleus but may not necessarily ascend to the cochlear nucleus or to other structures. The projections we observed emphasize the heterogeneity of the pathway origins as well as the complexity in the distribution of endings.

Axonal arborizations arose from fibers of 1–2 μm in diameter. Once within the cochlear nucleus, these fibers exhibited en passant swellings and branches along their course. The swellings were distinct, expanding to 3 μm in diameter but without any particular pattern to their occurrence. The branches tended to run in parallel to the main fiber after a short distance. The tips of the branches were characterized by distinct terminal swellings called boutons. En passant swellings and bouton endings were distributed throughout the cochlear nucleus, and their main difference seems to have been whether they marked the termination of a collateral. Occasionally, a larger, lobulated swelling appeared, giving rise to fine hairs and terminal swellings. The appearance of these lobulated endings from the spinal trigeminal nucleus resembled those generated by cuneate projections called mossy fibers (Wright and Ryugo, 1996; Wolff and Künzle, 1997). In the cochlear nucleus, these trigeminal mossy fibers were found only in the GCD.

The similarity of projections from the spinal trigeminal nucleus when compared to those of the cuneate nucleus (Wright and Ryugo, 1996) meant that we needed to confirm that we had not inadvertently involved the dorsal column system. In these cases, injections in the spinal trigeminal nucleus did not produce anterograde or retrograde labeling in the dorsal column nuclei. Labeled fibers and terminals were distributed within the medial but not the lateral division of the ventral posterior nucleus of the thalamus. The differential thalamic projection in these cases was consistent with involvement of the spinal trigeminal nucleus and not the dorsal column nuclei (Peschanski, 1984; Vienante et al., 2000).

It is important to consider the nature of retrograde neuronal labeling, especially in light of data that show cells in nonauditory and unexpected structures (e.g., Fig.

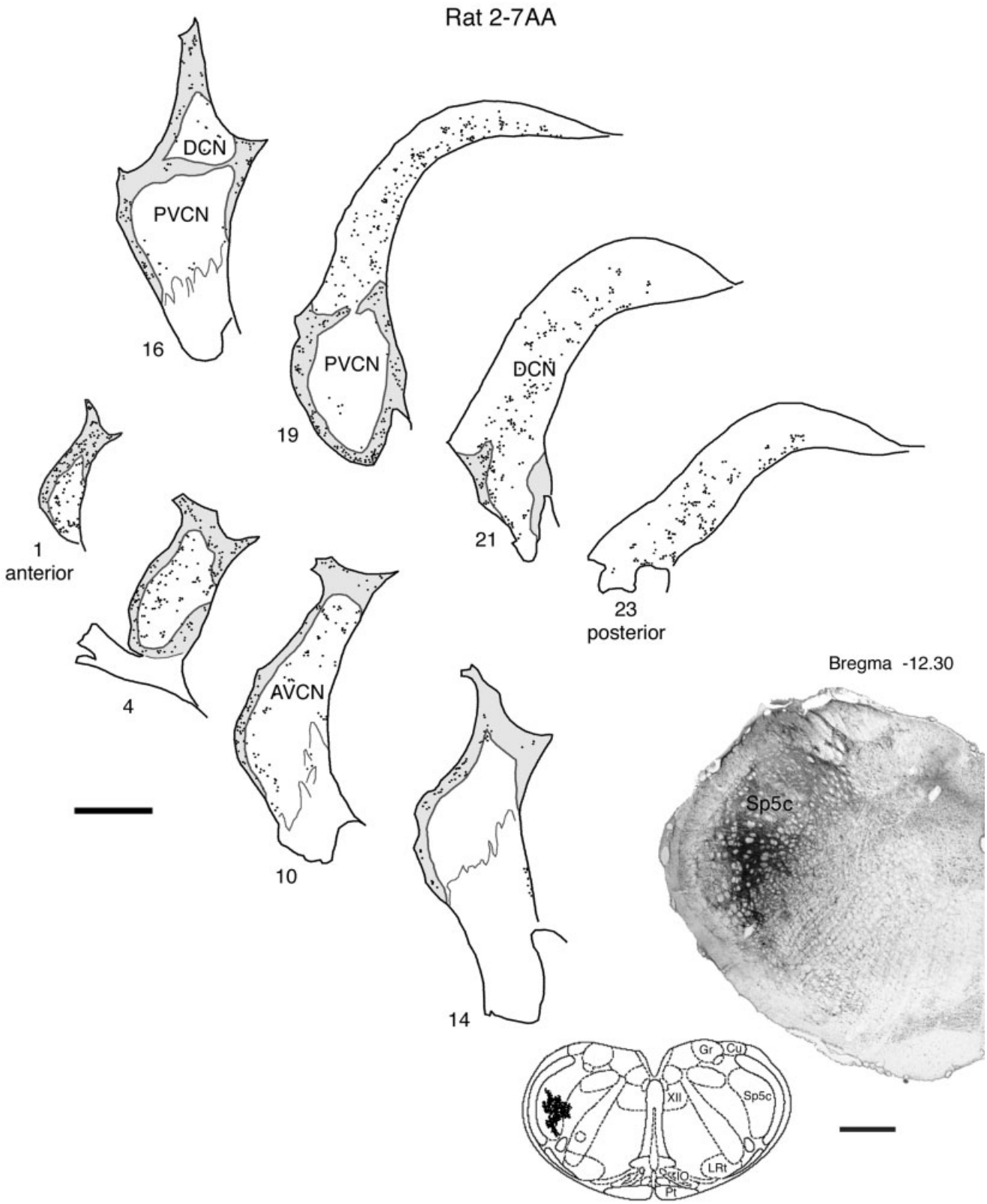

Fig. 5. Plot shows distribution of labeled endings from Rat 2-7AA with an injection of BDA confined to the ventral region of the spinal trigeminal nucleus, *pars caudalis*. A photomontage through the injection site along with a drawing is shown in the lower right. Representative sections are shown, with anterior sections having low numbers and the GCD indicated in gray. Each black dot represents a single swelling. Note that most of the endings are distributed within the GCD and the deep DCN; there is also a projection to the AVCN, and this feature is related to the ventral injection site. AVCN, anteroventral cochlear nucleus; Cu, cuneate nucleus; DCN, dorsal cochlear nucleus; Gr, gracile nucleus; IO, inferior olive; LRt, lateral reticular nucleus; Pt, pyramidal tract; PVCN, posteroventral cochlear nucleus; Sp5c, spinal trigeminal nucleus *pars caudalis*; XII, hypoglossal nucleus. Scale bars = 0.5 mm.

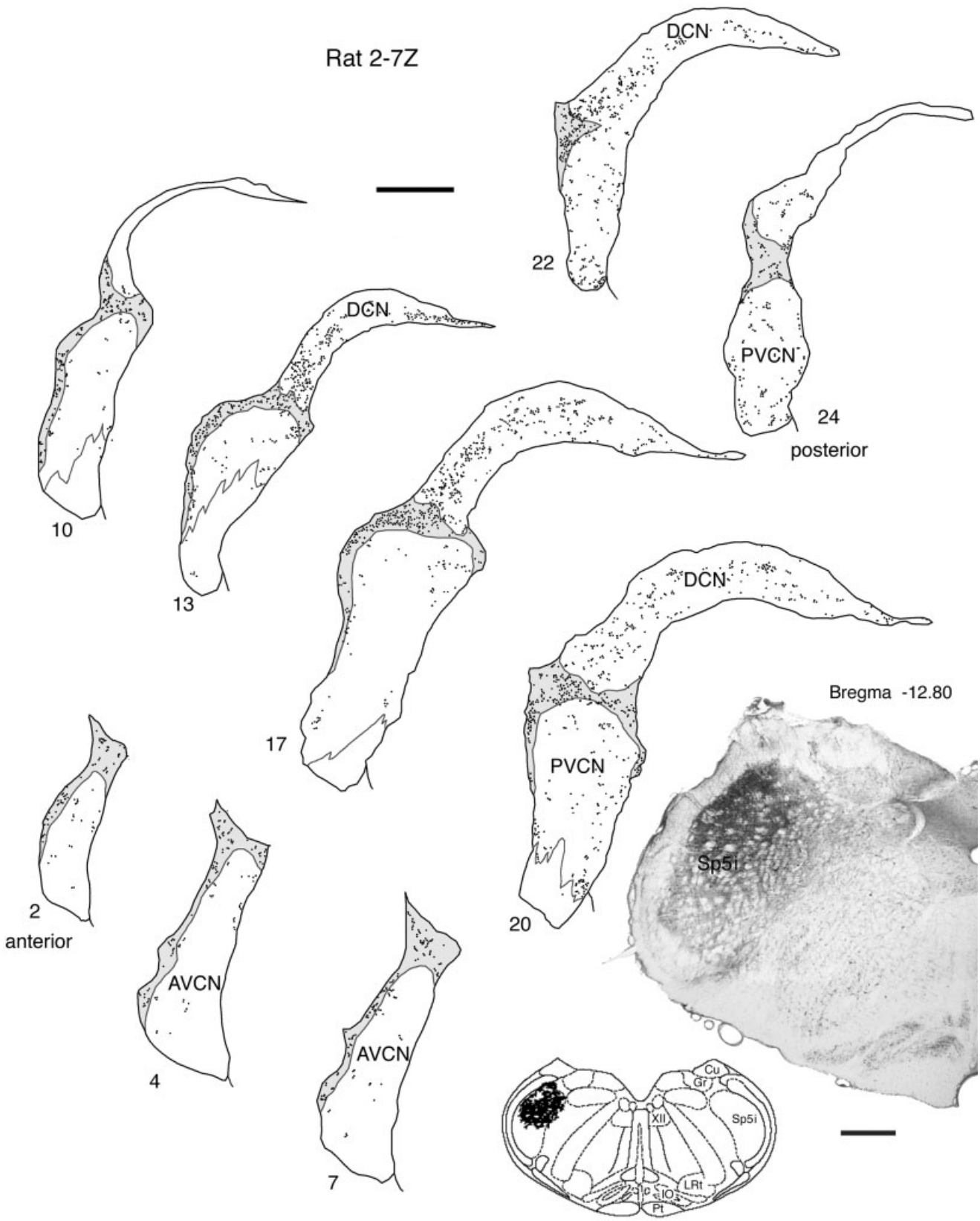

Fig. 6. Plot shows labeled endings from Rat 2-7Z with an injection of BDA confined to the more dorsal region of the spinal trigeminal nucleus, *pars interpolaris*. A photomontage through the injection site along with a drawing is shown on the lower right. Representative sections are shown, with anterior sections having low numbers and the GCD indicated in gray. Each black dot represents a single swelling. Most of the terminals are distributed within the GCD and deep DCN. Terminals are evident in the PVCN, typical of the more dorsal injection site. AVCN, anteroventral cochlear nucleus; Cu, cuneate nucleus; DCN, dorsal cochlear nucleus; Gr, gracile nucleus; IO, inferior olive; LRN, lateral reticular nucleus; Pt, pyramidal tract; PVCN, posteroventral cochlear nucleus; Sp5i, spinal trigeminal nucleus *pars interpolaris*; XII, hypoglossal nucleus. Scale bars = 0.5 mm.

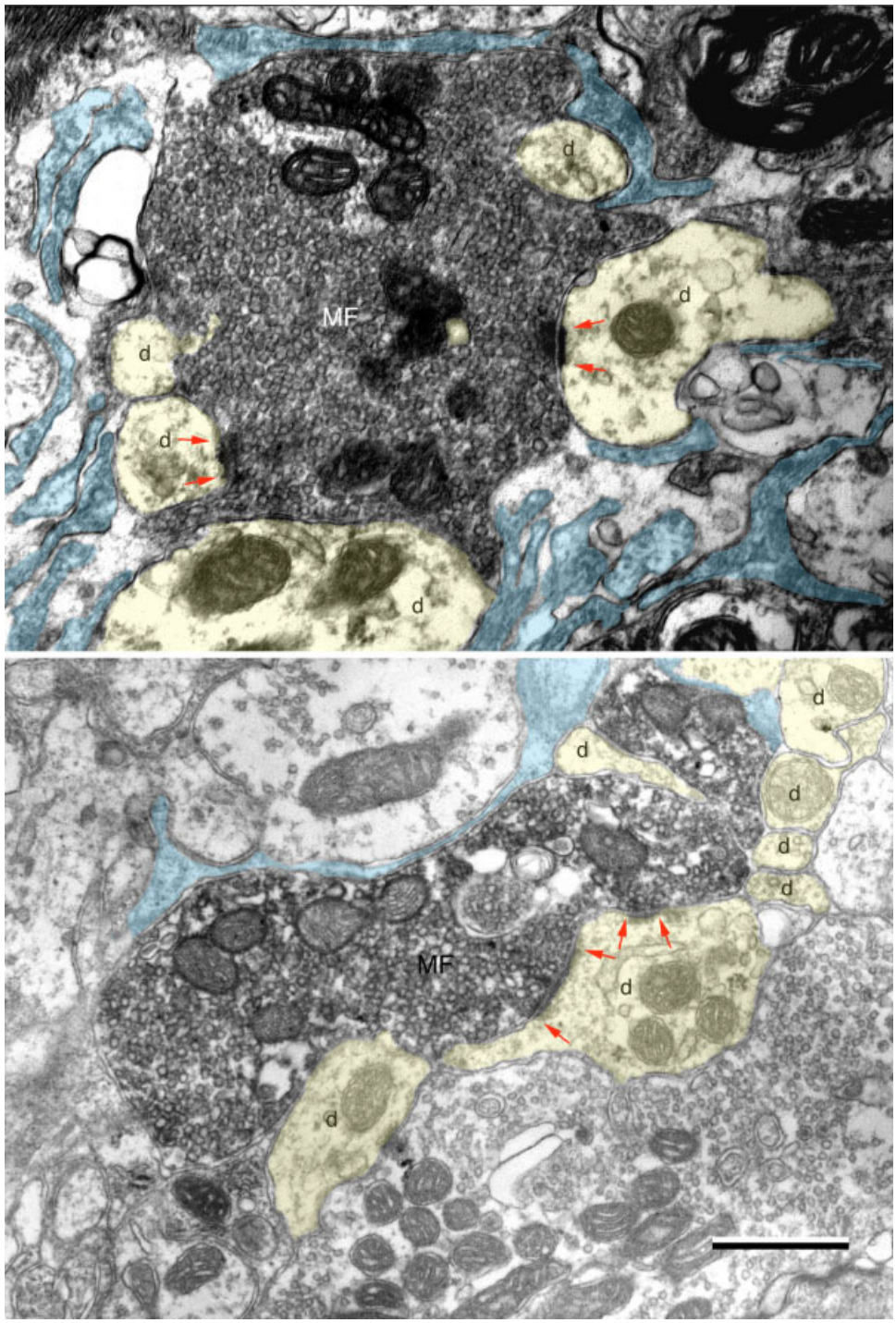

Fig. 7. Electron micrographs of representative mossy fibers (MF) labeled with BDA. Mossy fibers are filled with round synaptic vesicles and exhibit prominent postsynaptic membrane thickenings (flanked by red arrows). The associated presynaptic membrane exhibits presynaptic projections of electron dense material. The perimeter of the ending is studded by profiles of granule cell dendrites (shaded light yellow and labeled "d"). Glial lamellae are shaded blue. The structural characteristics of individual synapses are similar regardless of origin from a bouton, en passant ending, or mossy fiber. Scale bar 0.5 μm.

2). Our injections of Fast Blue or BDA into the cochlear nucleus were performed with the overlying cerebellar tissue aspirated and all sites were histologically confirmed to be contained within the nucleus. There was no label on the surface of the brain stem or cerebellum that would indicate diffusion away from the injection site. Microscopic examination failed to detect labeled fibers in adjacent tracts, including the middle and inferior cerebellar peduncles, the vestibular nerve root, and the descending tract of the trigeminal nerve, suggesting that the dye did not spread and create a "fibers of passage" problem. We have been concerned that labeling of cells in precerebellar structures reflected staining due to contamination. Yet the literature has numerous instances where unconventional connections are found with the auditory system. These connections include the somatosensory brain stem nuclei (Itoh et al., 1987; Weinberg and Rustioni, 1987; Wolff and Künzle, 1997), primary afferents of the spinal

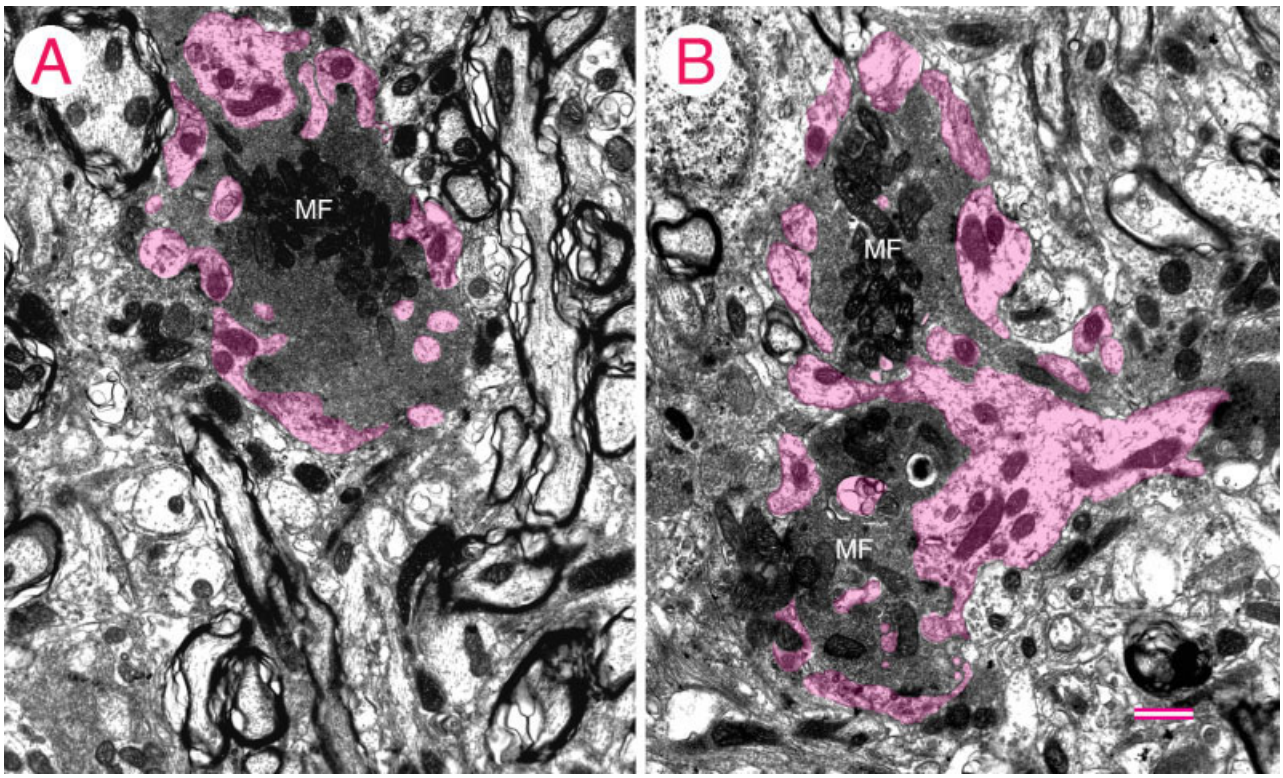

Fig. 8. Electron micrographs show representing mossy fibers in relation to granule cell dendrites (tinted purple). Single mossy fiber (**A**) and pair of mossy fibers (**B**) form the central core of the synaptic complex, incompletely surrounded by profiles of granule cell dendrites. Fine hair-like appendages extend from the granule dendrites to penetrate the mossy fiber, characteristic of the relationship. The dendrites and the appendages can serve as synaptic targets. Scale bar = 1 μm.

cord (Pfaller and Arvidsson, 1988), the dorsal raphé nucleus (Ye and Kim, 2001), and the pontine nuclei (Ohlrogge et al., 2001). In our experiments involving auditory pathways, we have consistently observed labeled cells in "nontraditional" structures. Hearing is a highly complicated process and we hypothesize that the connections we have shown between the cochlear nucleus and other structures are real.

A projection has been described from the peripherally located trigeminal ganglion into auditory brain stem structures (Shore et al., 2000). These authors described trigeminal nerve projections to a marginal area called the small cell cap and to the magnocellular core of the VCN; no mention was made of projections to granule cells. They also used retrograde methods to label trigeminal ganglion cells following dye injections in the VCN. Although our injections in the cochlear nucleus did not reveal labeling in the trigeminal ganglion, this difference could be due to our small injections that were centered near the granule cell lamina and the DCN. Our results are consistent with the observation that the trigeminal ganglion projects to the VCN but not to the DCN (Shore et al., 2000). It should be noted, however, that species differences cannot be ruled out.

Spinal trigeminal endings

The spinal trigeminal projection to the cochlear nucleus terminates as en passant swellings, terminal boutons, and mossy fibers. These endings all formed synaptic specializations that featured round synaptic vesicles and associated asymmetric densities. The postsynaptic targets were usually dendrites. Most of the targets were thin profiles characteristic of granule cell dendrites that also exhibited prominent pre- and postsynaptic membrane densities. Mossy fibers occupied a central core around which dendrites formed a terminal tuft. Thin appendages emerged from the terminal dendrites and penetrated into the mossy fiber. These appendages also served as postsynaptic targets.

Although we did not follow these dendrites through serial sections back to an identified cell type, the synaptic relationship resembled that between mossy fibers and the terminal dendritic claw of granule cells (Wright and Ryugo, 1996; Weedman et al., 1996). The GCD, however, is also filled with distinct classes of microneurons that have different dendritic morphologies (Mugnaini et al., 1980b, 1994; Floris et al., 1994; Mugnaini and Floris, 1994; Weedman and Ryugo, 1996; Wright and Ryugo, 1996; Hutson and Morest, 1997; Doucet and Ryugo, 1997). The unipolar brush cell (UBC) emits a single thick dendritic stalk that branches profusely at the tip. A single mossy fiber is completely enclosed by a complex nest of these UBC dendrites. In contrast, the chestnut cell receives axosomatic synapses onto its cell body from many mossy fibers. Spinal trigeminal projections did not engage in these other types of synaptic relationships. Mossy fibers from the contralateral pontine nuclei contacted the shafts of larger dendrites (Ohlrogge et al., 2001).

Mossy fiber terminals in the cochlear nucleus are found in the GCD and form complex synaptic structures with granule cell dendrites, Golgi cell axons, and surrounding encapsulating glial elements (Mugnaini et al., 1980a; Wright and Ryugo, 1996; Weedman et al., 1996). The spinal trigeminal mossy fibers resembled mossy fibers arising from the cuneate nucleus (Wright and Ryugo, 1996). These somatosensory mossy fibers are filled with round synaptic vesicles and make identical asymmetric synapses. The cuneate mossy fibers are immunoreactive to glutamate but not GABA, glycine, or choline acetyltransferase (Wright and Ryugo, 1996). We have not repeated the antibody staining for spinal trigeminal inputs to the CN, but the structural similarity in the projections suggests identical excitatory action. It is striking that unlabeled mossy fibers far outnumbered the labeled ones. The implication is that there are still unknown sources of mossy fiber inputs to the GCD.

Spinal trigeminal nucleus

The projecting neurons appear to be a diverse group of multipolar neurons with respect to somatic size and shape. There is a tendency for these neurons to be distributed on the lateral side of the nucleus but this observation does not constitute a rule. Labeled neurons could be found in all parts of the nucleus. The spinal trigeminal nucleus has been considered to have three parts: a superficial marginal layer, the substantia gelatinosa, and the deeplying magnocellular zone. The projections neurons are said to lie in the marginal layer and magnocellular zone (Gobel, 1974). Because the fluorescent tracer Fast Blue did not completely stain individual neurons with their dendrites and spines, however, it is beyond the scope of this article to reconcile our observations with those who studied cell types (e.g., Ramón y Cajal, 1909; Gobel, 1974).

The spinal trigeminal nucleus is one of three central nuclei that comprise the general somatic afferent column of the trigeminal sensory system. This column also includes the principal sensory nucleus and the mesencephalic nucleus of the trigeminal. The spinal trigeminal nucleus extends from the pons to the upper cervical spinal cord and has three distinct subdivisions along its rostrocaudal extent: *pars oralis, pars interpolaris*, and *pars caudalis*. It is thought that each of these subdivisions has a different function (Matesz, 1983; Shigenaga et al., 1986; Hallas and Jacquin, 1990; Wang et al., 1994). The cell bodies of the afferent nerve fibers are mostly located in the ipsilateral trigeminal ganglion, also known as the Gasserian ganglion. Three branches of the nerve, ophthalmic, maxillary, and mandibular, are laid out in a topographic fashion, terminating respectively in ventral, middle, and dorsal longitudinal columns (Wall and Taub, 1962; Henry and Westrum, 1990; Florence and Lakshman, 1995; Dessem et al., 1997). They carry proprioceptive, discriminative touch, nociceptive, and thermal information from the face, scalp, and oral and nasal mucosa. It is thought that the *pars caudalis* is responsible for temperature sensibility and nociception (McHaffie et al., 1994; Wang et al., 1994). The subdivision *pars interpolaris* receives mechanoreceptor inputs that conceivably convey information about eyelid, pinna, and superficial muscle position (van Ham and Yeo, 1996; May and Porter, 1998; Kanold and Young, 2001). The subdivision *pars oralis* receives inputs predominantly from the mucous membranes of the mouth, nose, and eye but also proprioceptive afferents from muscles of mastication and vibrissae (Gibson, 1987; Olsson et al., 1988; Gong et al., 2003). These neurons provide extensive local interconnections among the other trigeminal sensory nuclei as well as project to specific nuclei in the thalamus, brain stem motor and sensory nuclei, inferior olive, cerebellum, and superior colliculus (Hockfield and Gobel, 1982; Hallas and Jacquin, 1990; Li et al., 1993; Jiang et al., 1997; Pinganaud et al., 1999; Buisseret-Delmas et al., 1999; Craig, 2003). Our results demonstrate that the cochlear nucleus needs to be added to this list of targets, and reveal that a study of afferent convergence upon the projection cells (to the cochlear nucleus) is needed in order to sort out what kinds of somatosensory information is being delivered to the auditory system.

Functional considerations

There is a direct pathway from the spinal trigeminal nucleus to the cochlear nucleus (cat, Itoh et al., 1987; rat, Weinberg and Rustioni, 1987; Wright and Ryugo, 1996; hedgehog, Wolff and Künzle, 1997). Injections of WGA-HRP into C1-C3 of the dorsal root ganglia in rats consistently labeled a small projection to the AVCN, whereas injections into the trigeminal ganglion did not (Pfaller and Arvidsson, 1988). One interpretation for this auditory-somatosensory interaction was proposed in phylogenetic terms because of the close relationship between the dorsal column nuclei and the lateral-line system in phylogenetically old amphibians (Weinberg and Rustioni, 1987). The idea stems from a consideration of the lateral-line system as a transducer for sound as well as for touch. Once these systems for processing sound and touch became established in primitive animals, they would presumably resist change over time without the influence of selective pressure (Nikundiwe and Nieuwenhuys, 1983). Other ideas involved synergistic enhancement between sensory systems (Itoh et al., 1987), mechanisms by which to make the auditory system less sensitive to self-generated body noises such as muscle contractions or joint movements (Wright and Ryugo, 1996; Ryugo et al., 2003), or keeping the animal apprised of pinna position involved in head-related transfer functions (Oertel and Young, 2004).

A series of double-labeling studies in the rat showed that somatosensory neurons could project to multiple targets by way of axon collaterals. Neurons of the *pars caudalis* and *interpolaris* of the spinal trigeminal nuclei and the dorsal column nuclei projected simultaneously to both the cochlear nucleus and the external inferior colliculus (Li and Mizuno, 1997a) as well as to the cochlear nucleus and the ventrobasal thalamus (Li and Mizuno, 1997b). The projecting fibers simultaneously provide somatosensory signals to multiple brain stem nuclei so that information pertaining to head and pinna position might be integrated across modalities.

Electrophysiological experiments have shown functional consequences of somatosensory stimulation on auditory responses. In decerebrate cats with cervical spinal cuts that spared only the dorsal columns, dorsal column activation bilaterally modulated sound-evoked discharges of neurons of the DCN (Saadé et al., 1989). Single units recorded in the vicinity of the DCN/PVCN boundary were excited by electrical stimulation in the somatosensory nuclei (trigeminal and dorsal column nuclei), some of which also responded to sound (Young et al., 1995). Analysis of the evoked potentials produced by such electrical stimulation suggested that the somatosensory inputs activated

cochlear granule cells projecting to the DCN molecular layer. The story, however, is not complete. Stimulation of the pinna, especially rotation of the auricle was particularly effective in inhibiting DCN neurons, whereas stimulation of the face and vibrissae had no effect (Kanold and Young, 2001). The physiological data are only partially consistent with anatomical observations concerning somatosensory inputs to the cochlear nucleus (Itoh et al., 1987; Weinberg and Rustioni, 1987; Pfaller and Arvidsson, 1988; Wright and Ryugo, 1996; Wolff and Künzle, 1997). It remains to be determined whether these apparent conflicts are a result of combining data from different species.

The GCD of the cochlear nucleus receives a broad array of inputs from different sources. Some of these inputs arrive in the form of mossy fiber endings. This mix of input is conveyed by granule cell parallel fibers to cartwheel and pyramidal cells of the DCN (Mugnaini et al., 1980b; Manis, 1989; Weedman et al., 1996). The relationship of granule cells to the DCN has an organization resembling that of a cerebellar folium and the electrosensory nuclei of some fish and amphibians (Mugnaini and Morgan, 1987; Wright and Ryugo, 1996; Devor, 2000; Ryugo et al., 2003; Oertel and Young, 2004). Electrosensory structures such as the dorsal octavolateral nucleus use "sensory subtraction" to separate extrinsic environmental signals from self-generated signals (Montgomery and Bodznick, 1994; Bell et al., 1997, 1999). Might the GCD-DCN circuits serve to subtract self-generated sounds such as chewing, respiration, and heart beats? The involvement of the GCD in comparing actual with anticipated auditory information could also have definite implications in learning mechanisms. Does pinna position fit into the scheme of "predicted information"? Cerebellar-like sensory structures could mediate neuronal mechanisms that subtract learned predictions about sensory input from actual sensory input to make unpredicted sensory events more salient. The data presented in this report will help establish the structural substrate that might underlie multimodal processing within the auditory system.

Clinical implications

There have been several reports of certain tinnitus patients that can modulate their phantom auditory perception through voluntary actions of their jaw or by adopting particular neck positions (Sanchez et al., 2002; Levine et al., 2003). This phenomenon is known as somatic or craniocervical tinnitus. A neurological model aimed at explaining these observations speculates that these tinnitus patients may present pathologic somatosensory-auditory neural connections within the central nervous system (Levine, 1999). The present data are related to this clinical phenomenon and may provide clues to its treatment.

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