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CUNEATE AND SPINAL TRIGEMINAL NUCLEUS PROJECTIONS TO THE COCHLEAR NUCLEUS ARE DIFFERENTIALLY ASSOCIATED WITH VESICULAR GLUTAMATE TRANSPORTER-2

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Abstract—There are distinct distributions and associations with vesicular glutamate transporters (VGLUTs) for auditory nerve and specific somatosensory projections in the cochlear nucleus (CN). Auditory nerve fibers project primarily to the magnocellular areas of the ventral cochlear nucleus and deepest layer of the dorsal cochlear nucleus and predominantly colabel with VGLUT1; whereas the spinal trigeminal nucleus (Sp5) projections terminate primarily in the granule cell domains (GCD) of CN and predominantly colabel with VGLUT2. Here, we demonstrate that the terminals of another somatosensory pathway, originating in the cuneate nucleus (Cu), also colabel with VGLUT2. Cu projections in cochlear nucleus exhibited a bilateral distribution pattern with ipsilateral dominance, with 30% of these classified as putative mossy fibers (MFs) and 70% as small boutons (SBs). Cu anterograde endings had a more prominent distribution in the GCD than Sp5, with a higher percentage of MF terminals throughout the CN and higher MF/SB ratio in GCD. 56% of Cu endings and only 25% of Sp5 endings colabeled with VGLUT2. In both cases these were mostly MFs with only 43% of Cu SBs and 18% of Sp5 SBs colabeled with VGLUT2. The few Cu and Sp5 terminals that colabeled with VGLUT1 (11% vs. 1%), were evenly distributed between MFs and SBs. The high number of VGLUT2-positive Cu MFs predominantly located in the GCD, may reflect a faster-acting pathway that activates primarily dorsal cochlear nucleus cells via granule cell axons. In contrast, the higher percentage of Sp5-labeled SB terminals and a greater number of projections outside the GCD suggest a slower-acting pathway that activates both dorsal and ventral cochlear nucleus principal cells. Both projections, with their associations to VGLUT2 likely play a role in the enhancement of VGLUT2 after unilateral deafness [Zeng C, Nannapaneni N, Zhou J, Hughes LF, Shore S (2009) J Neurosci 29:4210–4217] that may be associated with tinnitus.

Key words: VGLUTs, cuneate nucleus, spinal trigeminal nucleus, cochlear nucleus, tinnitus.

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Abbreviations: BDA, biotinylated dextran-amine; CN, cochlear nucleus; Cu, cuneate nucleus; DCN, dorsal cochlear nucleus; GCD, granule cell domain; MF, mossy fiber; PBS, phosphate-buffered saline; SB, small boutons; Sp5, spinal trigeminal nucleus; VCN, ventral cochlear nucleus; VGLUT, vesicular glutamate transporter.

The cochlear nucleus (CN) is the first central auditory nucleus receiving inputs from not only the receptor cells of the cochlea via the auditory nerve, but also from a number of somatosensory nuclei, including the spinal trigeminal nucleus (Sp5), trigeminal ganglion and cuneate nucleus (Cu) (Haenggeli et al., 2005; Itoh et al., 1987; Zhou and Shore, 2004; Shore et al., 2000; Weinberg and Rustioni, 1987). These somatosensory inputs are ultimately conveyed to CN principal cells and are involved in multimodal integration with auditory nerve projections to these cells (Zhan and Ryugo, 2007; Zhou and Shore, 2004; Shore, 2005). These non-auditory projections are mostly confined to the granule cell domain (GCD) of the CN (Zhou et al., 2007; Zhan and Ryugo, 2007), which includes both the superficial shell region of the ventral CN (VCN) and the fusiform cell layer of dorsal cochlear nucleus (DCN) and contains numerous small cells, including granule cells (Mugnaini et al., 1980a,b; Hackney et al., 1990; Weedman et al., 1996; Zhou and Shore, 2004).

The cuneate and gracile nuclei together form the dorsal column nuclei, which receive somatosensory inputs from dorsal root ganglion cells that innervate touch, vibratory, and proprioceptive receptors on the body surface (Itoh et al., 1987; Weinberg and Rustioni, 1987, 1989; Young et al., 1995). The projection from the Sp5 to the CN, on the other hand, conveys information about touch, vibration and proprioception from receptors in and around the vocal tract (Haenggeli et al., 2005; Shore and Zhou, 2006). Both the dorsal column and Sp5 pathways are involved in multimodal information processing at an early stage in the auditory pathway, but the types and distributions of terminal projections of the Cu versus Sp5 pathways in the CN remain unclear, thus the specific functional significance of each pathway is yet to be unraveled.

Vesicular glutamate transporters (VGLUTs) package glutamate into synaptic vesicles and serve as excellent markers of glutamatergic projections. The subtypes VGLUT1 and VGLUT2 have distinct distributions in the CN (Fremeau et al., 2004; Herzog et al., 2001; Takamori et al., 2001; Kaneko et al., 2002) and the distinct distributions of auditory and non-auditory projections to the CN are associated with specific VGLUTS. Auditory nerve fibers, which project primarily to the magnocellular areas of the ventral cochlear nucleus (VCNm) and deep layer of the dorsal cochlear nucleus (DCN3) (Brown and Ledwith, 1990) exclusively colabel with VGLUT1; whereas spinal trigeminal nucleus (Sp5) projections predominantly colabel with VGLUT2 (Zhou et al., 2007; Zeng et al., 2009). Gómez-Nieto and Rubio (2009) have also shown that VGLUT2-

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labeled endings of unknown origin contact the dendrites of bushy cells in the rat GCD region.

Although there are indications that the cuneo-cochlear nucleus pathway is glutamatregic (Wright and Ryugo, 1996), the VGLUT transporter involved in this pathway is still unknown. This is particularly important in light of recent findings that VGLUT2 density (Zhou et al., 2007) is increased after unilateral deafness, while VGLUT1, associated with auditory nerve fibers, is decreased (Zeng et al., 2009). This implies that projections that co-label with VGLUT2 are enhanced following deafness. Until now, only the Sp5 endings have been shown to co-label with VGLUT2.

The aim of this study was to determine whether Cu projections to CN, like those from Sp5, co-label with VGLUT2. Furthermore, we wished to compare the distributions and types of endings from each projection site to help elucidate the differential functions of each pathway. By using anterograde tract-tracing methods combined with VGLUT immunohistochemistry, the present study examined the differential distributions of terminals from Cu and Sp5 to the CN in guinea pigs and their association with VGLUT isotypes.

EXPERIMENTAL PROCEDURES

Animal preparation

Seven female pigmented guinea pigs with normal Preyer's reflexes (250–350 g, Elm Hill Breeding Labs, Chelmsford, MA, USA) were used in this study. All animals were anesthetized with i.m. injections of ketamine hydrochloride (Hospira, Inc., Lake Forest, IL, USA; 80 mg/kg) and xylazine (Shenandoah, IA, USA; 4 mg/kg) and placed in a stereotaxic frame (David Kopf, Tujunga, CA, USA). Rectal temperature was monitored and maintained at 38±0.5 °C with a thermostatically controlled heating pad. Lidocaine was infiltrated around the surgical area of the scalp, and a longitudinal incision was made after the animal was areflexic to a paw pinch and had no corneal reflex. All procedures were carried out in accordance with NIH guidelines for the care and use of laboratory animals (NIH publication No. 80-23), and guidelines provided by the University of Michigan (UCUCA protocol # 08539).

Four guinea pigs were used to identify the locations of labeled terminals from the Cu. Two of these were used to identify the colocalization of VGLUT1 and VGLUT2 with anterograde terminals from the Cu. Similarly, three guinea pigs were used to identify the locations of labeled terminals from the Sp5, two of which were used for VGLUT colocalization. The surgical procedure for injections of anterograde tracers in Sp5 has been described previously (Zhou and Shore, 2004). The procedure for Cu injections was similar. An opening just lateral-caudal to lambda was drilled in the occipital bone. A Hamilton microsyringe equipped with a glass micropipette (20–30 μm tip) was positioned into the left cuneate nucleus using stereotaxic coordinates (1.8 mm lateral to the midline, 3 mm caudal to the posterior edge of transverse sinus, and 8.8 mm ventral to the surface of the dura). A total volume of 0.1 μl anterograde tracer [10% biotinylated dextran-amine (BDA), MW 10,000, Molecular Probes] was pressure-injected into the Cu. After removing the pipette, the animals were sutured and allowed to recover for 6 or 7 days before being euthanized with FatalPlus (Vortech Pharmaceuticals, Dearborn, MI, USA; 5 mg/kg, i.p.) and perfused transcardially with 100 ml of 0.1 M phosphate-buffered saline (PBS; pH 7.4), followed by 400 ml of 4% paraformaldehyde in the same buffer. The brains were isolated and postfixed for 2 h at 4 °C, then transferred into 20% sucrose in 0.1 M phosphate buffer overnight at 4 °C. The brainstems were processed as described below for anterograde labeling.

Tissue processing and immunocytochemistry

The brainstems were sectioned with a freezing microtome at a thickness of 40 μm. Alternate sections were mounted in serial order on clean glass slides and air dried, yielding four equal series. One series was used for labeling BDA and VGLUT1, one was used for BDA and VGLUT2, and one was used for BDA only labeling to check the distribution of projection terminals in CN. To visualize BDA-labeled Sp5 and Cu terminals colabeled with VGLUT1 and VGLUT2, sections were incubated for 2 h with Cy2 conjugated with streptoavidin (1:300; Jackson ImmunoResearch, West Grove, PA, USA), followed by immunolabeling with VGLUT1 and VGLUT2.

The VGLUT1 and VGLUT2 immunocytochemistry procedure has been described previously (Zeng et al., 2009). Briefly, all tissue processing was done at room temperature (20–22 °C). Sections were incubated for 30 min in a blocking solution containing 1% normal goat serum in 0.1 M PBS with 0.1% Triton X-100, pH 7.4, followed by overnight incubation with primary antibodies, VGLUT1 (polyclonal antibody, generated in rabbit, diluted in

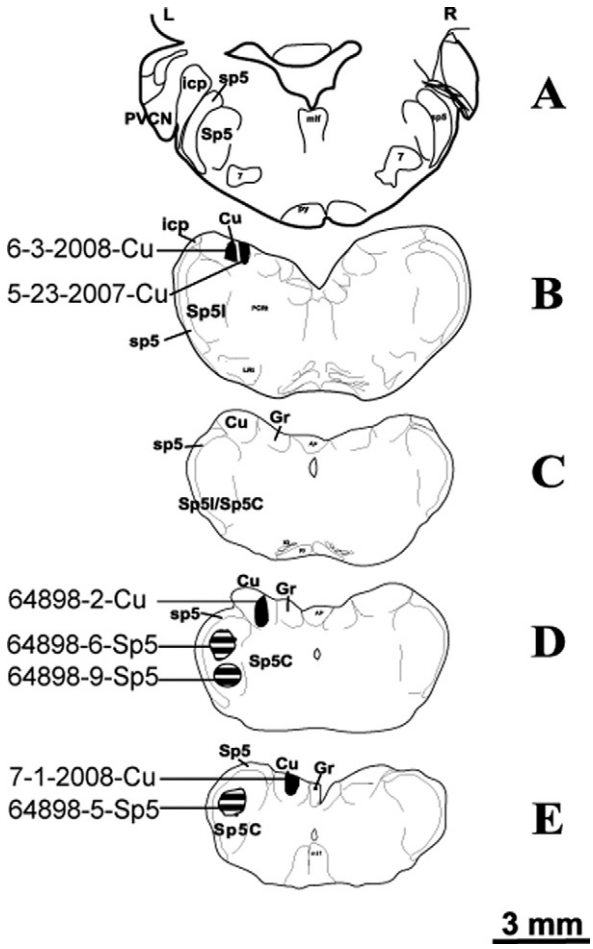

Fig. 1. Drawings of rostral (A)-to-caudal (E) transverse sections of the brain stem. The shaded areas represent four Cu injection sites (solid) and three Sp5 (cross hatched) injection sites. Note all four Cu injections are smaller than the Sp5 injections. Sp5, spinal trigeminal nucleus; sp5, spinal trigeminal tract; Sp5I, pars interpolaris of Sp5; Sp5C, pars caudalis of Sp5; icp, inferior cerebellar peduncle; Cu, cuneate nucleus; Gr, gracile nucleus; PVCN, posteroventral cochlear nucleus.