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REVEALING THE MOLECULAR LAYER OF THE PRIMATE DORSAL COCHLEAR NUCLEUS

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Abstract—In nonprimate mammals, the dorsal cochlear nucleus (DCN) is thought to play a role in the orientation of the head toward sounds of interest by integrating acoustic and somatosensory information. Humans and higher primates might not use this system because of reported phylogenetic changes in DCN cytoarchitecture [Moskowitz N (1969) Comparative aspects of some features of the central auditory system of primates. Ann N Y Acad Sci 167:357–369; Moore JK, Osen KK (1979) The cochlear nuclei in man. Am J Anat 154:393–418; Moore JK (1980) The primate cochlear nuclei: loss of lamination as a phylogenetic process. J Comp Neurol 193:609–629]. In this study, we re-evaluated this question from a comparative perspective and examined the rhesus monkey (cercopithecoid primate) using more sensitive probes and higher resolution imaging methods. We used electron microscopy to identify parallel fibers and their synapses, and molecular markers to determine that primates exhibit the main components of excitatory neurotransmission as other mammals. We observed that characteristics of the monkey molecular layer resembled what has been reported for nonprimates: (1) immunohistochemistry revealed many unmyelinated, thin axons and en passant glutamatergic synapses on dendritic spines; (2) immunohistochemistry for phosphodiesterase (PDE10A) showed the nuclei of granule cells distributed in the external molecular layer and the deep layers in the DCN; (3) antibodies for the inositol trisphosphate receptor (IP3r) and calbindin immunostained cartwheel cells; (4) postembedding immunogold labeling revealed synaptic expression of AMPA and delta glutamate receptor subunits on spines in parallel fiber endings; and (5) parallel fibers use vesicular glutamate transporter 1 (VGLUT1) to package glutamate into the synaptic vesicles and to mediate glutamate transport. These observations are consistent with the argument that the rhesus monkey DCN has neuronal features similar to those of other nonprimate mammals.

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Abbreviations: DCN, dorsal cochlear nucleus; IP3r, inositol 1,4,5-triphosphate receptor; LR, large round synaptic vesicle ending; MR, medium round synaptic vesicle ending; PB, phosphate buffer; PDE10A, phosphodiesterase 10A; PF, parallel fiber; PSD, postsynaptic density; SR, small round synaptic vesicle ending; VGLUT, vesicular glutamate transporter.

Key words: spines, glutamate receptors, VGLUT1, postembedding immunogold labeling, morphometry, ultrastructure.

The dorsal cochlear nucleus (DCN) receives direct input from the cochlea by way of the auditory nerve (Sando, 1965; Osen, 1970; Fekete et al., 1984). In nonprimate mammals, the cellular organization of the DCN is thought to play an important role in the orientation of the head toward sounds of interest by integrating acoustic and somatosensory information (May, 2000; Young and Davis, 2002; Ryugo et al., 2003; Shore, 2005). Whether this is also the case in man and higher primates is unclear because of reports that the DCN and associated granule cell regions undergo pronounced phylogenetic changes. Studies of the human cochlear nuclei described a complete and selective loss of the superficial layers of the nuclei, whereas deeper regions of the nuclei maintained the basic cytoarchitecture plan of mammals (Moskowitz, 1969; Moore and Osen, 1979; Heiman-Patterson and Strominger, 1985).

A transitional stage of the DCN granule cell system has been suggested for nonhuman primates because of its position between the full laminar development of the cochlear granule cell system in nonprimate mammals and its complete absence in humans (Moskowitz, 1969; Moore, 1980; Heiman-Patterson and Strominger, 1985). If granule cells are lost and not merely reorganized or relocated, then higher primates would have to replace integrating mechanisms for multimodal sensory inputs that occur in the superficial layers of the DCN. This loss would be most apparent by the absence of granule cells and their parallel fiber synapses (PF) on the apical dendrites of fusiform and cartwheel cells.

Studies in the DCN of nonprimate mammals have shown that the synapses of PF on fusiform cells and cartwheel cells express a unique composition of glutamate receptors and specific types of computational capabilities (Petralia et al., 1996; Rubio and Wenthold, 1997; Gardner et al., 1999, 2001; Fujino and Oertel, 2003; Tzounopoulos et al., 2004, 2007). This feature would allow PF synapses to differ in their electrical properties and plasticity potential with their neuronal target. In the auditory system, plasticity at synapses is important for adaptation to normal fluctuations in the sensory environment (Molitor and Manis, 1997; Turecek and Trussell, 2000; Fujino and Oertel, 2003), such as learning to recognize the calls of a new troop member and perhaps the processing of abnormal auditory input (Kaltenbach et al., 2005; Illing and Reisch, 2006). Therefore the comparative question regarding the superficial DCN with its synaptic circuitry was re-evaluated in order to better understand the substrates of normal hearing, deaf-

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ness, tinnitus, and conditions where cochlear implants are needed.

In this study, we examined the rhesus monkey (cercopithecoid primate) to determine whether the superficial DCN of higher primates has similar synaptic components as nonprimate mammals. To do so, we made use of 1) ultrastructure to characterize PFs and their synapses, and 2) molecular markers to determine whether primates exhibit the main components of excitatory neurotransmission. The data argue that the DCN of rhesus monkeys has similar neuronal features as nonprimate mammals.

EXPERIMENTAL PROCEDURES

Tissue procedure

Tissue from rhesus monkeys was obtained after transcardial perfusion at the Johns Hopkins Medical School, Baltimore, MD, USA, or the Emory Primate Center, Atlanta, GA, USA. At both institutions, animal protocols were approved by the institutional animal care and use committees and followed NIH guidelines. All experiments conformed to named local and international guidelines on the ethical use of animals. This research minimized the number of animals used and their suffering.

Three rhesus monkeys (*Macaca mulatta*) of 3 years of age were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Brains were removed and postfixed overnight with the same fixative. Brainstems were sectioned with a Vibratome and stored in 0.2 M PB. Some of the brainstem slices were kept in a cryoagent solution until use, others were postfixed in 2% glutaraldehyde in 0.1 M PB pH 7.2 for 4 h, washed with 0.1 M PB for 30 min, washed in 0.1 M cacodylate buffer pH7.2 and postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 45 min.

Three additional rhesus monkeys (*Macaca mulatta*), 6, 9, and 11 years of age were perfused with 4% paraformaldehyde and a low percentage of glutaraldehyde (e.g. 0.1 or 0.5% glutaraldehyde) in 0.1 M PB. Brains were removed and immersed in buffer without postfixation. Brainstems were sectioned with a Vibratome at 70 or 300 μm in thickness. The thin sections were maintained in a cryoagent solution at −20 °C until used. The 300 μm sections were cryoprotected in glycerol and followed the freeze-substitution procedure (see below).

Conventional transmission electron microscopy

Brainstem slices were dehydrated through a series of ethanol solutions (50%, 70%, 85%, 95% and 100%), infiltrated with epoxy resins and flat embedded. Blocks with cochlear nuclei were trimmed and mounted on blocks and cut with an ultramicrotome. Ultrathin sections (80 nm in thickness) were counterstained with uranyl acetate and lead citrate and analyzed with a TECNAI G2 Spirit Biotwin TEM (FEI, Hillsboro, OR, USA). The images were captured with an AMT XR40 4 megapixel side mounted CCD camera (Danvers, MA, USA). Image processing was performed with Adobe Photoshop using only the brightness and contrast commands.

Free floating and preembedding immunocytochemistry

Vibratome slices followed two cycles of freezing and thawing on dry ice, washed in 0.1 M PB and were either stained for Nissl or incubated with the corresponding primary antibody [calbindin-D28k (1:3000, Sigma, St. Louis, MO, UA), IP3r (inositol 1,4,5-triphosphate receptor; 0.1 μg/ml, Sharp et al., 1993; Ryugo et al., 1995); PDE10A (phosphodiesterase 10A; 1.2 μg/ml, Seeger et al., 2003); GluR1 (1.5 mg, Wenthold, 1992; Douyard et al., 2007), GluR2/3 (1.5 mg; Wenthold, 1992; Douyard et al., 2007), GluR4 (1.5 mg, Wenthold, 1992; Douyard et al., 2007), delta [1/2] (1.0 mg/ml; Mayat et al., 1995; Rubio and Wenthold, 1997), and vesicular glutamate transporter (VGLUT) 1 (1:2000; Synaptic System, Göttingen, Germany)] in 0.1 M PB for 48 h at 4 °C. All the antibodies used were polyclonal with the exception of calbindin and PDE10A. Slices were blocked for 1 h with 10% normal goat or horse serum in buffer and followed the standard protocol for diaminobenzidine detection as previously described (Ryugo et al., 1995; Rubio and Wenthold, 1997). Nickel was added to intensify the immunoreaction of calbindin, VGLUT1 and some slices immunostained for delta [1/2]. Some of the sections were mounted on glass slides, air dried and covered for further analysis with an Olympus BX51 research microscope. Other sections were postfixed for 1 h in 1% osmium tetroxide, washed and embedded in EPON as described above. Ultrathin sections (80 nm in thickness) were counterstained with uranyl acetate and lead citrate and analyzed with a TECNAI G2 Spirit Biotwin TEM. The images were captured with an AMT CCD camera and processed with Adobe Photoshop using only the brightness and contrast commands.

Freeze-substitution and postembedding immunogold labeling

For the detection of GluR1, GluR2/3 and GluR2, AMPA receptor subunits, delta [1/2] and VGLUT1 with immunogold labeling after freeze-substitution, a protocol similar to that described in detail elsewhere was used (Rubio and Wenthold, 1997, 1999; Rubio, 2006). The DCN was dissected and processed for freeze-substitution and low-temperature embedding. For postembedding immunocytochemistry, ultrathin sections (80 nm in thickness) on nickel grids were incubated in sodium borohydride and glycine in Tris-buffered saline solution with Triton X-100. After being preblocked with serum, the sections were incubated with affinity purified primary antibodies for GluR1 (1.5 μg), GluR2/3 (1.5 μg), the monoclonal antibody against GluR2 N-terminus (1.5 μg; Chemicon, Temecula, CA, USA; Rubio 2006), delta [1/2] (1 μg), or VGLUT1 (1:300) for single postembedding immunogold labeling. Primary antibodies were detected with secondary antibodies conjugated to 5 nm gold particles in diameter (1:20; Amersham GE Healthcare, Buckinghamshire, UK). No gold particles were observed at the postsynaptic density (PDS), synaptic endings of inhibitory synapses, or on mitochondria and myelin sheets. Controls sections were prepared either in the absence of the primary antibody during the incubation step or by preadsorption of GluR1, GluR2/3 and GluR2 antibodies with the corresponding peptides (Rubio and Wenthold, 1997, 1999; Matsui et al., 2005). No gold particles were observed on the ultrathin sections after any of the control procedures. The analysis of the localization of two proteins (GluR1 and GluR2 or GluR2 and VGLUT1) on the section was done using double post-embedding immunogold labeling and using antibodies conjugated to gold particles of two different sizes. Ultrathin sections were analyzed with a TECNAI G2 Spirit Biotwin TEM. The images were captured with an AMT CCD camera at 49,000× or 68,000× magnification. Image processing was performed with Adobe Photoshop using only the brightness and contrast commands to enhance gold particles.

Morphometric analysis of synaptic endings and vesicles

Morphometric analysis of synaptic endings and synaptic vesicles in the 3 and 9-year-old rhesus monkey was performed on electron micrographs using the ImageJ software. ImageJ is a public domain, Java-based image processing program developed at the National Institutes of Health (available at: http://rsb.info.nih.gov/ij/download.html). Parameters of the synaptic vesicles included: area, major and minor diameter, and circularity (where a ratio of 1 indicates round and smaller ratios indicate a progressively flatter structure). The size of the vesicle was represented by its approx-