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ULTRASTRUCTURE, SYNAPTIC ORGANIZATION, AND MOLECULAR COMPONENTS OF BUSHY CELL NETWORKS IN THE ANTEROVENTRAL COCHLEAR NUCLEUS OF THE RHESUS MONKEY

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Abstract—Bushy cells (BCs) process auditory information in the ventral cochlear nucleus (VCN). Yet, most neuroanatomical findings come from studies in cats and rodents, and the ultrastructural morphological features of BCs in humans and higher nonhuman primates are unknown. In this study, we combined histological, immunocytochemical, and ultrastructural methods to examine the morphology and synaptic organization of BCs in the rhesus monkey VCN. We observed that BCs were organized in a complex neural network that appears to interconnect the cells. The fine structure of BC somata and dendrites, as well as their synaptic inputs, are similar to those in other mammals. We found that BCs received numerous endbulb-like VGLUT1- and VGLUT2-immunopositive endings. In addition, they expressed glutamate AMPA (GluR2/3 and GluR4), NMDA (NR1), delta1/2 receptor subunits, and the α1 subunit of the glycine receptor. These receptor types and subunits mediate fast excitatory synaptic transmission from the cochlea and inhibitory neurotransmission from noncochlear inputs. Parvalbumin immunostaining and semithin sections showed that BC dendrites are oriented toward neighboring BC somas to form neuronal clusters. Within the cluster, the incoming inputs established multiple, divergent synaptic contacts. Thus, BCs were connected by specialized dendrosomatic and somasomatic membrane junctions. Our results indicate that the cytoarchitectural organization of BCs is well conserved between primates and other mammalian species.

Key words: cochlear nucleus, electron microscopy, gap junctions, immunohistochemistry, synaptic integration, synchronization.

Bushy cells (BCs) of the anteroventral cochlear nucleus (AVCN) are responsible for the precise temporal processing of acoustic information. The firing of BCs is determined by the interaction between glutamatergic, GABAergic, and glycinergic inputs (Kopp-Scheinpflug et al., 2002). The physiological properties are consistent with evidence that the BC soma and dendrites receive excitatory and inhibitory inputs from many sources (Saint Marie et al., 1986; Wu and Oertel, 1986; Schofield, 1994; Mahendrasingam et al., 2004; Spirou et al., 2005; Gómez-Nieto and Rubio, 2009).

Two types of BCs—spherical and globular—have been described in nonprimate mammals. They differ in size, ultrastructural properties of the cell body, and projection patterns (Cant and Morest, 1979a,b; Cant and Casseday, 1986; Tolbert and Morest, 1982; Tolbert et al., 1982; Spirou et al., 2005; Cao et al., 2007). Large synaptic complexes from the auditory nerve fibers—the endbulbs and modified endbulbs of Held—innervate the cell body of all BC types and ensure the high-fidelity transmission of auditory fiber activity (Pfeiffer, 1966; Brawer and Morest, 1975; Liberman, 1991; Sento and Ryugo, 1989; Ryugo and Sento, 1991). BCs express AMPA-selective glutamate receptors (GluR3 and 4) that have the fastest gating and desensitization kinetics (Wang et al., 1998; Gardner et al., 2001; Kemmer and Vater, 2001).

BCs are also identified by a prominent dendritic tree that forms a complex tufted pattern of arborization (Cant and Morest, 1979a; Tolbert et al., 1982; Rouiller and Ryugo, 1984; Gómez-Nieto and Rubio, 2009). Thus, BC dendrites provide space for many compartmentalized excitatory and inhibitory synaptic interactions from cochlear and noncochlear origins (Ostapoff and Morest, 1991; Gómez-Nieto and Rubio, 2009). Further, BC dendrites cluster with other BCs to form a neuronal network in which BCs are linked by direct neuron-neuron connections and receive divergent multisynaptic contacts from afferent inputs (Gómez-Nieto and Rubio, 2009).

During recent decades, tremendous progress has been made in understanding the morphological basis of auditory information processing by BCs. Yet, there is little neuroanatomical information on BCs in primates. Here, we investigate whether the most relevant morphological findings of BCs also exist in the rhesus monkey (cercopithecoid primate). In particular, we focus on the ultrastructural distinctions of BCs, their expression of neurotransmitter receptors, and the axon terminals that synapse on the somata and dendrites. We find that the BC network described in rats (Gómez-Nieto and Rubio, 2009) also exists in higher primates. Our study suggests that BCs in the rhesus monkey and other mammals are homologous with regard to their ultrastructure, afferent input, receptor expression, and organization within the AVCN.

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Abbreviations: AVCN, anteroventral cochlear nucleus; BCs, bushy cells; GBCs, globular bushy cells; GluR, glutamate receptors; SBCs, spherical bushy cells.

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EXPERIMENTAL PROCEDURES

Animals and tissue procedure

Brain tissue from rhesus monkeys was provided by Drs. David Ryugo and Yoland Smith after transcardial perfusion at Johns Hopkins Medical School, Baltimore, MD, USA, and the Emory Primate Center, Atlanta, GA, USA. At both institutions, animal protocols were approved by institutional animal care and use committees and followed NIH guidelines. All experiments conformed to local and international guidelines on the ethical use of animals, and all efforts were made to minimize the number of animals that was used and their suffering.

Four rhesus monkeys (*Macaca mulatta*), aged 6–11 years, were perfused with 4% paraformaldehyde, ($n=2$ for light microscopy) with or without 0.1% glutaraldehyde in 0.1 M phosphate buffer (PB) ($n=2$ for electron microscopy). Brains were removed and postfixed overnight with the same fixative; the samples for electron microscopy were postfixed with 2% glutaraldehyde in 0.1 M PB, pH 7.2 for 48 h.

Brainstems were sectioned in the coronal or sagittal plane (60- and 100-μm thickness for light and electron microscopy, respectively) with a Vibratome (#VT1000S, Leica, Vienna, Austria). Selected sections that contained the caudal and rostral regions of AVCN were examined by immunohistochemistry or electron microscopy (see below).

Tissue preparation for electron microscopy

Brainstem slices of two rhesus monkeys perfused with glutaraldehyde were transferred to cacodylate buffer (0.1 M, pH 7.2) and osmicated for 2 h in 1% osmium tetroxide (#RT-19100; Electron Microscopy Science, Fort Washington, PA, USA). After several washes in distilled water, sections were dehydrated in a series of ethanols (50%, 70%, 85%, 95%, and 100%), infiltrated with epoxy resin (#EMbed-812; Electron Microscopy Science), and embedded flat between acetate sheets (Rubio et al., 2008).

Sections were examined under a brightfield microscope, and only those that contained the AVCN (from caudal to rostral regions) were selected, trimmed, and mounted onto EMbed 812 blocks, from which semithin (0.5 μm) and serial ultrathin sections (silver-gold interference ~70 nm) were sliced with a Leica Ultramicrotome. Semithin sections for light microscopy were placed onto microscope slides and stained with Toluidine Blue. Ultrathin sections were collected onto Formvar-coated single slot grids and counterstained with Uranyl Acetate and Lead Citrate.

Antibody characterization

In this study we used immunohistochemistry (see below) with specific primary antibodies (Table 1) to determine the expression of vesicular glutamate transporters, parvalbumin and specific subunits of ionotropic glutamate and glycine receptors in the AVCN of the rhesus monkey.

By Western blot of guinea pig cerebellum and cochlear nucleus, the rabbit polyclonal antibodies for VGLUT1 and VGLUT2 recognized a single band at ~60 kDa and ~65 kDa, respectively (Zhou et al., 2007). Preincubation of VGLUT antibodies with corresponding synthetic peptides (Table 1) resulted in negative immunolabeling in guinea pig brainstem sections (Zhou et al., 2007).

Table 1. Primary antibodies used

Antigen	Host	Immunogen	Manufacturer	Dilution
Vesicular glutamate1 transporter (VGLUT1)	Rabbit	Strep-Tag fusion protein containing amino acid residues 456–560 of rat VGLUT1	Synaptic Systems (Göttingen, Germany), rabbit polyclonal, #135 302	1:1000
Vesicular glutamate 2 transporter (VGLUT2)	Rabbit	Strep-Tag fusion protein containing amino acid residues 410–582 of rat VGLUT2	Synaptic Systems (Göttingen, Germany), polyclonal, #135 402	1:1000
Vesicular glutamate 2 transporter (VGLUT2)	Mouse	Recombinant protein from rat VGLUT2	Chemicon (Temecula, CA, USA), monoclonal, #MAB5504	1:1500
Parvalbumin (PV)	Mouse	Purified IgG1 isotype parvalbumin from frog muscle and directed against an epitope at the first calcium-binding site and specifically stains the calcium-bound form of parvalbumin (from manufacturer's technical information)	Chemicon (Temecula, CA, USA), monoclonal, #MAB1572 lot No. LV1505479	1:1500
GluR2/3 AMPA receptor subunits	Rabbit	Synthetic peptide (EGYNVYGIESVKI) corresponding to the C-terminus 13 amino acids of the rat GluR2/3 subunit	Polyclonal Ab T73, gift from Robert Wenthold (Wenthold et al., 1990)	1:1000
GluR4 AMPA receptor subunit	Rabbit	Synthetic peptide (RQSSGLAVIASDLP) corresponding to the C-terminus 14 amino acids of the rat GluR4 subunit	Polyclonal Ab F73, gift from Robert Wenthold (Wenthold et al., 1990)	1:1000
NR1 NMDA receptor subunit	Rabbit	Synthetic peptide (LQNQKDTVLPRRAIEREEGQLQLCSRHRES; amino acids 909–938) corresponding to the C-terminus of the rat NR1 subunit	Chemicon (Temecula, CA, USA); polyclonal, #AB1516	1:1000
Delta 1/2 glutamate receptor subunit	Rabbit	Synthetic peptide (QPTPTLGLNLGNDPDRGTSI) corresponding to the C-terminus 20 amino acids of the rat glutamate δ2 subunit	Polyclonal Ab T25, gift from Robert Wenthold (Mayat et al., 1995)	1:1000
Glycine receptor alpha 1 subunit (GlyRα1)	Mouse	Purified GlyRα1 peptide from rat spinal cord, (epitope corresponding to the N-terminus 96–105 residues of the rat α1-subunit) (Schröder et al., 1991). This epitope is highly conserved in all α subunits and the β subunit (Grenningloh et al., 1987, 1990; Kuhse et al., 1990; Harvey and Betz, 2000)	Monoclonal Ab 4a Alexis Biochemicals (San Diego, CA, USA), #804-037-C100	1:1500