Published in final edited form as:

Neuroscience. 2011 April 14; 179: 188–207. doi:10.1016/j.neuroscience.2011.01.058.

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 $\alpha 1$ 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.

Keywords

cochlear nucleus; electron microscopy; gap junctions; immunohistochemistry; synaptic integration; synchronization

INTRODUCTION

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

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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.

MATERIALS AND METHODS

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 to 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- μ m 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 2 rhesus monkeys perfused with glutaraldehyde were transferred to cacodylate buffer (0.1 M, pH 7.2) and osmicated for 2 hours in 1% osmium tetroxide (#RT-19100; Electron Microscopy Science, Fort Washington, PA). 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 μ m) and serial ultrathin sections (silver-gold interference $\sim$ 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 $\sim$ 60 kDa and $\sim$ 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).

The mouse monoclonal antibody MAB5504 (Table 1) was raised against recombinant protein from rat VGLUT2 and has shown species reactivity to mouse and rat. The immunogen for this antibody was the entire VGLUT2 protein (information obtained from the manufacturer). The antibody has not been epitope-mapped by Chemicon; thus, the epitope for MAB5504 is unknown. By Western blot of mouse brain lysates, immunostained VGLUT2 has a predicted molecular weight of 60 kDa (as shown by a comparable product from Abcam Cambridge, MA). Double immunolabeling of the monoclonal (MAB5504) and polyclonal (#135 402) antibodies against VGLUT2 generated the same pattern and labeled the same synaptic endings in the rat cochlear nucleus (Gómez-Nieto and Rubio, 2009).

The monoclonal antibody against parvalbumin (MAB1572; Table 1) reacts with parvalbumin from brain and muscle. By immunoblot of mouse brain lysates it recognizes a single band of 12 kDa. According to the manufacturer, this antibody does not respond with other members of the EF-hand family. No preadsorption studies have been reported for this antibody.

Rabbit polyclonal antibodies (Table 1) against GluR2/3, GluR4, and delta 1/2 (1.0 mg/ml) were kindly provided by Dr. Robert Wenthold and have been previously characterized (Wenthold et al., 1990; Mayat et al., 1995; Rubio and Wenthold, 1997; 1999). Antibodies were generated against synthetic peptides that corresponded to sequences from the glutamate

receptor (GluR) subunits GluR2-4 (Table 1). Following conjugation to bovine serum albumin (BSA) using glutaraldehyde and injection into rabbits (Wentholt et al. 1990), the antibodies were affinity-purified using the peptide, which was attached to activated CH Sepharose 4B (Pharmacia LKB Biotechnology, Piscataway, NJ). The specificity of the antibodies was determined by Western blot of membranes of simian kidney cells (COS-7) that were transfected with GluR subunit DNA and rat brain homogenates. The antibodies recognized a single band at 108 kDa (Wentholt et al., 1990; Rubio and Wentholt, 1999).

The rabbit polyclonal antibody against delta1/2 was generated against a C-terminal peptide of the glutamate receptor delta 2 subunit (Table 1) and recognizes delta 1 and delta 2 but not AMPA, kainate, NMDA, or the mGluR1 α glutamate receptor subunits, based on Western blot analysis of transfected HEK-293 cells. Western blot analysis of the rat brain showed a single immunoreactive band at 114 kDa (Mayat et al., 1995). Displacement of immunoreactivity by antigenic peptide was also measured to establish the specificity of the AMPA and delta antibodies (Mayat et al., 1995; Rubio and Wentholt, 1997, 1999).

The rabbit polyclonal antibody against NR1 (#AB1516, Chemicon) and mouse monoclonal antibody against GlyR α 1 (Alexis Biochemicals, San Diego, CA; #804-037-C100; now part of Enzo Life Sciences, Plymouth, PA) (Table 1) were obtained from commercial sources. The polyclonal antibody against NR1 recognizes a peptide that corresponds to the C-terminus of the rat NMDAR1 C1 splice variant (manufacturer's technical information; see also Monyer et al., 1992). Western blot analysis of rat brain homogenates revealed the expected band at 120 kDa (Moriyoshi et al., 1991; Petralia et al., 1994) and showed no crossreactivity with other glutamate receptor subtypes (Petralia et al., 1994).

The monoclonal antibody against GlyR α 1 (Table 1) was obtained by immunization with affinity-purified native GlyR from rat spinal cord. Antibody specificity was established in HEK293T cells transfected with cDNAs of each GlyR α subunit (Schröder et al., 1991; Haverkamp et al., 2004). In Western blots of adult and neonatal rat spinal cord, the antibody recognized the 48-kDa and 58-kDa isoforms of the glycine receptor. In homogenates of young adult (P30) rat cochlear nuclei, a single band was observed at approximately 48 kDa (data not shown). Using postembedding immunolabeling, monoclonal anti-GlyR α 1 labeled the postsynaptic membrane only of inhibitory synapses (Jusuf et al., 2005).

Immunohistochemistry

Free-floating brainstem sections followed two cycles of freezing and thawing on dry ice, were washed in 0.1 M PBS and incubated with the corresponding primary antibody (Table 1) as described previously (Gómez-Nieto et al. 2008b; Rubio et al., 2008; Gómez-Nieto and Rubio, 2009). Briefly, all immunostaining steps were performed at room temperature (~22°C) unless otherwise stated. Washes and dilutions of antisera were made in 0.1 M phosphate-buffered saline (PBS), pH 7.4. Slices were blocked for 1 h with 10% normal goat or horse serum in buffer and were incubated with primary antibodies for 18–24 h at 4°C. Sections were washed and followed an incubation with the corresponding biotinylated secondary antibody for 1 hr (eliteVectastain ABC immunolabeling; VectorLabs Burlingame, CA). After, the immunoreaction was visualized with diaminobenzidine (VectorLabs) (Rubio et al., 2008). Nickel was added to intensify the immunoreactions of parvalbumin, VGLUT1, VGLUT2, and certain immunostains of delta1/2 and GlyR α 1. All sections were mounted onto glass slides, air-dried, and coverslipped with Permount (Fisher Scientific, Fair Lawn, NJ) for analysis by light microscopy.

In addition, to examine the codistribution of VGLUT1 and VGLUT2 on bushy cells, we performed double immunofluorescence for the 2 isoforms. For this experiment, we followed

a protocol by Gómez-Nieto and Rubio (2009). We used a monoclonal antibody against VGLUT2 (mouse anti-VGLUT2; #MAB5504; Chemicon, Temecula, CA; now part of Millipore, Billerica, MA) with a polyclonal antibody against VGLUT1. As secondary antibodies, we used Cy3-goat anti-mouse (#115-165-166; Jackson ImmunoResearch, West Grove, PA) and Cy5-goat anti-rabbit (#115-175-003; Jackson ImmunoResearch). The sections were rinsed, mounted onto slides, and coverslipped with ProLong® Antifade Kit (#P7481; Molecular Probes) for confocal microscopy analysis. Omission of the primary antibody resulted in the absence of immunostaining. Because VGLUT immunoreactivity is well documented in the cerebellar cortex (Takamori et al., 2001), we used the cerebellum as the positive control (data not shown).

Light microscopy and image analysis

Sections that were processed for light microscopy were examined on an upright brightfield microscope (Olympus BX-50) equipped with a digital camera. Low-magnification images of the AVCN and cerebellum were taken with the 4x or 10x objective. High-resolution images were acquired with a 40x or 100x objective lens (oil immersion) for morphometric analysis of the histological structures of interest. We estimated the size (area in μm^2) of the VGLUT1- and VGLUT2-labeled terminals ($n = 110$) and the length and thickness of the labeled dendrites and axons with ImageJ (version 1.39e-Java 1.5.0, NIH; <http://rsb.info.nih.gov/ij>).

Sections that were processed for confocal microscopy were analyzed on a Leica TCS SP2 confocal laser scanning microscope (Leica Microsystems, Mannheim, Germany) coupled to a Leica DM IRE2 inverted microscope equipped with argon and helium neon lasers (excitation wavelengths of 458, 476, 488, 543, 568, and 633 nm). The fluorochromes Cy3 or Cy5 were detected sequentially, stack by stack, with an acousto-optical tunable filter system and the triple dichroic mirror TD488/543/633, using laser lines at 546 and 633 nm, respectively.

Electron microscopy and image analysis

Sections that were processed for electron microscopy were examined on a transmission electron microscope (TECNAI G2 Spirit Biotwin, TEM, FEI Hillsboro, OR, USA; JEOL1400, Musashino 3-chome Akishima Tokyo 19, Japan), coupled to a side-mounted CCD camera (AMT XR40 megapixel; Denvers MA, USA and Orius SC200-830 780 Gatan Inc., Commonwealth Drive Warrendale, PA, respectively). Digital electron micrographs were taken at magnifications from $\times 2900$ to $\times 68,000$.

The spherical and globular bushy cells in the primate AVCN were identified using criteria that have been used to describe both BC types in other mammals (Cant and Morest, 1979a, b; Cant, 1981; Tolbert et al., 1982; Wang et al., 1998; Mahendrasingam et al., 2004; Asako et al., 2005; Gómez-Nieto and Rubio, 2009).

In addition, we performed a subsequent characterization of the identified cell bodies as follow. Each of the photographed cell bodies was given a unique code from 001 to 122 ($N=122$). We then classified each cell body with the following criteria: a) the position within the AVCN: from caudal to rostral; b) the shape of the cell body: round, spherical/ovoid or other; c) the position of the nucleus: central or eccentric; d) the existence of smooth nuclear membrane; e) the presence of excitatory input on the cell body: endbulb, modified-endbulb or none; f) the existence of endoplasmic reticulum stacks oriented concentrically in the cytoplasm around the nucleus or the existence of fine and dispersed endoplasmic reticulum within the cytoplasm; and g) the size of the cell body (small, large or giant).

Morphometric analyses—A morphometric analysis of the BC somata was also performed to identify any differences in size with regard to area and major diameter. Of 122 cell bodies that were photographed from the caudal and rostral regions of the AVCN, 45 were identified as BCs. These BC cell bodies were silhouetted and measured using ImageJ (version 1.39e-Java 1.5.0, NIH). These neurons shared the described criteria for bushy cells. Only cell bodies sectioned through the nucleolus were included in the analysis.

A morphometric analysis of the synaptic endings and synaptic vesicles in rhesus monkey was also performed on electron micrographs. One thousand four hundred ten synaptic vesicles of 47 terminals (30 vesicles per terminal) were measured with ImageJ. The selection of the synaptic vesicles was random but limited to vesicles with clearly visible plasma membranes. Asymmetric and symmetric synapses were identified using criteria that were developed by Gray (1959). We also considered qualitative features for the classification, such as the electron-dense appearance of the axoplasm and synaptic vesicles and packing density of synaptic vesicles in the synaptic terminals (Rubio and Juiz, 2004; Gómez-Nieto and Rubio, 2009).

Parameters of the synaptic vesicles included area, minor and major diameter of the vesicles, and circularity (wherein a ratio of 1 indicates roundness and smaller ratios indicate progressively flatter structures) (Rubio and Juiz, 2004; Rubio et al., 2008; Gómez-Nieto and Rubio, 2009). The size of the vesicle was represented by its approximate mean diameter, calculated by $[(\text{major axis} + \text{minor axis})/2]$.

To determine whether differences between 2 samples were statistically significant, we used Student's *t*-test with a P-value threshold of <0.01 . Two independent investigators performed blind morphometric and statistical analyses of the samples and obtained similar results. The data were then compared with the qualitative classification of the endings. The morphometric and qualitative analyses showed the same 5 distinct types of endings (3 with round vesicles and 2 with pleomorphic or flattened vesicles).

All photographs in the figures were processed and adjusted with minor modifications in brightness and contrast using Adobe Photoshop® (version 9.0; Adobe Systems Incorporated, San Jose, CA) and assembled using Canvas 7.0.

Identification of specialized membrane junctions—To distinguish between membrane specializations, we followed our criteria from an earlier report in rats (Sotelo et al., 1976; Gómez-Nieto and Rubio, 2009) and elsewhere (Henkart et al., 1976; Cant and Morest, 1979b; Landis and Reese, 1982; Mugnaini, 1985; Brunso-Bechtold and Vinsant, 1988; Rash et al., 1998). These membrane specializations include synaptic contacts, puncta adherentia, plasma membrane subsurface cisternae, and gap junctions. The length of the specialized membrane junctions were calculated with ImageJ.

RESULTS

Fine structure of bushy cells in the anteroventral cochlear nucleus of rhesus monkeys

To determine whether bushy cells (BCs) existed in the anteroventral cochlear nucleus (AVCN) of the rhesus monkey, we studied the ultrastructure of the caudal region near the root of the auditory nerve (Fig. 1A) and the most anterior region of the nucleus (Fig. 2A). As described in other mammals (Osen 1969; Brawer et al. 1974; Cant and Morest 1979a,b; Tolbert and Morest 1982; Tolbert et al. 1982; Cant and Casseday 1986), we identified cell bodies and primary dendrites that fit the ultrastructural categories of globular and spherical bushy cells (GBCs and SBCs, respectively) (Figs. 1, 2, 3). Both cell types were observed in both regions of the monkey AVCN. GBCs generally were located near the

root of the auditory nerve (Fig. 1), whereas SBCs were predominantly observed in the rostral region (Fig. 2).

Based on cell body size, we distinguished 2 subtypes: spherical and globular bushy cells (Fig. 3). Differences in cell body area and major diameter were statistically significant (Fig. 3C–D).

GBCs had predominantly round cell bodies with a major diameter of $28.6 \pm 3.2 \mu\text{m}$, an area of $474.7 \pm 24.3 \mu\text{m}^2$ and had a circularity of 0.95. They had eccentrically located nuclei, smooth nuclear contours, and prominent nucleoli. In the cytoplasm, GBCs had generally fine and dispersed rough endoplasmic reticulum (Nissl granules at the light microscopic level) (Figs. 1B, 3). Numerous tubular profiles of the Golgi apparatus were observed surrounding the nucleus. These ultrastructural properties were similar to those in cats (Osen 1969; Brawer et al., 1974; Cant, 1981).

SBCs were smaller than GBCs (major diameter: $24.8 \pm 1.5 \mu\text{m}$; area: $369.6 \pm 22.0 \mu\text{m}^2$) and were more ovoid in shape with a circularity of 0.90. These results were similar to those previously described for the putative spherical cells called “ovoid” by Heiman-Patterson and Strominger (1985) in a light microscopic study of the rhesus monkey AVCN. The nucleus of SBCs was more centrally located with a smooth nuclear contour. A prominent ultrastructural characteristic of SBCs was abundant rough endoplasmic reticulum cisterns. Stacks of rough endoplasmic reticulum were oriented concentrically in the cytoplasm (Fig. 2). A concentration of rough endoplasmic reticulum also was found surrounding the nuclear envelope, but it was not as prominent as reported previously in cats (Cant and Morest, 1979b).

As in cats and rodents (Saint Marie et al., 1986; Wu and Oertel, 1986; Schofield, 1994; Mahendrasingam et al., 2004; Spirou et al., 2005), both types of BCs had a cell body nearly encapsulated by synaptic endings that were characterized ultrastructurally as either excitatory or inhibitory (see below; Figs. 1, 2, 4).

In addition, we found that smaller and larger cell bodies were dispersed in the caudal region of the AVCN. None of these cell bodies shared the ultrastructural characteristics of GBCs and SBCs, and they were not included in the study.

GBCs and SBCs in the rhesus monkey had one or two primary dendrites that extended from opposite poles of the soma (Figs. 3E), similar to rodents and cats (Roullier and Ryugo, 1984; Ostapoff and Morest, 1991; Ryugo and Sento, 1991; Gómez-Nieto and Rubio, 2009). These dendrites were observed frequently in tangential semithin and ultrathin sections and branched into distal tufted dendrites $\sim 29 \pm 4.1 \mu\text{m}$ from the cell body. BC dendrites contained clumps of large mitochondria, abundant smooth endoplasmic reticulum, multivesicular bodies, and microtubules (Figs. 3F–H, 5).

Dendrites of BCs, covered by excitatory and inhibitory synaptic endings, were observed in the proximity of other BC somata (Fig. 5). As in rodents (Gómez-Nieto and Rubio, 2009), the dendrites were innervated by the endings that also synapsed on the cell bodies of neighboring BCs (see below).

Excitatory and inhibitory synaptic endings on bushy cells

We distinguished five types of synaptic endings on the cell bodies and dendrites of BCs (GBCs and SBCs) (Figs. 1, 2, 4, 5, 6). Three of them fitted the criteria of excitatory endings with asymmetric synaptic contacts and round vesicles (Figs. 1T1, 3G, 4, 5B–D). The other

two types were putative inhibitory endings with symmetrical synaptic contacts and pleomorphic or flattened synaptic vesicles (Figs. 1T2–3, 5E–F).

Ultrastructure of excitatory endings on BCs—Of the excitatory endings on GBC and SBC somata, one subtype had the ultrastructural characteristics of cochlear afferents (endbulb of Held) in cats and rodents (Cant and Morest, 1979b, Sento and Ryugo, 1989; Ryugo and Sento, 1991; Nicol and Walmsley, 2002; Spirou et al., 2005, Gómez-Nieto and Rubio, 2009). These endings were large ($\sim 8.5 \mu\text{m}^2$), made multiple asymmetrical synaptic contacts ($\sim 0.4 \mu\text{m}$ each)—some of which were curved toward the cytoplasm—with the soma and primary dendrite (Figs. 1T1, 4, 5B), and contained clear, round synaptic vesicles. These endings emerged from thick myelinated axons that displayed heminodes (Fig. 4), as reported in cats (Spirou et al., 2005).

In addition, we observed at least two other types of endings with clear, round synaptic vesicles that differed qualitatively from those of the auditory nerve. Neither of the other endings emerged from myelinated axons. One ending was larger ($\sim 18 \mu\text{m}^2$) and more irregular in shape than the endbulb, and it had larger synaptic vesicles (Figs. 3F, 5C). These endings usually made multiple asymmetric synaptic contacts ($\sim 0.3 \mu\text{m}$ each) on the soma and dendrites of BCs. The other ending was smaller ($\sim 1.8 \mu\text{m}^2$), made single asymmetric synaptic contacts ($\sim 0.25 \mu\text{m}$ length) and contained smaller synaptic vesicles (Fig. 5D).

To characterize these terminals, we performed a morphometric analysis of the shape (roundness) and size (area) of the synaptic vesicles (Fig. 6; Student's t-test, $P < 0.01$). We classified these endings as large (LRnd), medium (MRnd), or small (SRnd) based on the area of the vesicles (Fig. 6). Only MRnd vesicle-containing profiles shared the ultrastructural characteristics of the endbulb of Held. The profiles that contained the larger synaptic vesicles (LRnd) also were the largest; the profiles with the smallest synaptic vesicles (SRnd) were the smallest. All three types of excitatory endings were observed on both the proximal and distal dendrites of BCs (Figs. 5B–D; 6C).

Ultrastructure of inhibitory endings on BCs—The sources of putative inhibitory endings on BCs are intrinsic interneurons in the VCN, glycinergic interneurons in the dorsal cochlear nucleus, and descending inputs from upper auditory nuclei (Wu and Oertel, 1986; Schofield, 1994; Ostapoff et al., 1997). We distinguished 2 types of inhibitory endings on the somata and dendrites of GBCs and SBCs in rhesus monkeys (Figs. 1T2–3, 5E–F). These endings were similar in size ($\sim 2.5 \mu\text{m}^2$) and made single, symmetrical synaptic contacts ($\sim 0.5 \mu\text{m}$) on BCs, but the endings had differentially shaped synaptic vesicles. One contained pleomorphic (Pleo) vesicles, and the other type had flattened (F) vesicles. Our morphometric analysis of the synaptic vesicles confirmed the existence of 2 types of endings (Fig. 6; Student's t-test, $P < 0.01$).

Neurotransmitter receptor types in the primate bushy cells

As in rats, BCs in the rhesus AVCN expressed GluR2/3 and GluR4 AMPA receptor subunits, NR1 NMDA subunits, and the delta 1/2 receptor subunits (Fig. 7). All subunits were observed in BC somata and, to a lesser extent, along the dendrites. The labeling for GluR1 and GluR2 AMPA receptor subunits was weak and it was nearly indistinguishable from the myelinated regions (data not shown) that lacked immunostaining.

Glycine is the main inhibitory neurotransmitter in the cochlear nucleus (Altschuler et al., 1986, 1993; Caspary et al., 1994; Potashner et al., 1993; Rubio and Juiz, 2004). Our ultrastructural analysis showed that the rhesus BC somata and dendrites received inhibitory synaptic terminals that are probably glycinergic terminals (Figs. 1T2–3, 5C–D). As reported

in rodents (Sato et al., 1991), we found that the GlyR α 1 subunit was expressed widely throughout the rhesus cochlear nucleus and labeled BC dendrites and cell bodies (Fig. 7F).

Punctate VGLUT1 and VGLUT2 staining on BCs

Immunostaining for VGLUT1 and VGLUT2 showed that both isoforms were expressed in the rhesus cochlear nucleus (Fig. 8). In general, VGLUT1 was found in large endbulb-like puncta ($\sim 12.9 \mu\text{m}^2$) and small *en passant* endings (puncta $\sim 2.7 \mu\text{m}^2$) (Fig. 8A–B). Often, we also observed that the VGLUT1 puncta emerged from thick axonal segments ($\sim 3 \mu\text{m}$ in diameter). The VGLUT2 puncta, ranging from $\sim 3.9 \mu\text{m}^2$ to $\sim 18.6 \mu\text{m}^2$, were larger than those containing VGLUT1 (Fig. 8C–D), and they were distributed within both the center (core) and superficial regions of the nucleus. Small and large VGLUT1 and VGLUT2 puncta were distributed on BC-like somata and dendrites (Figs. 8A–D). In general, the large VGLUT1 puncta nearly encircled the perimeter of the cell body, whereas the large VGLUT2 puncta frequently were located at one pole of the soma. Double immunofluorescence for VGLUT1 and VGLUT2 showed that, as reported in the rat AVCN (Gómez-Nieto and Rubio, 2009), the isoforms did not overlap (data not shown).

Bushy cells form neuronal clusters in the anteroventral cochlear nucleus of rhesus monkeys

Our study in rodents using retrogradely-labeled BCs demonstrated that these neurons cluster within the AVCN (Gómez-Nieto and Rubio, 2009). Therefore, we wanted to determine whether primate BCs also formed clusters within the nucleus. In the present study, we were unable to use retrogradely-labeled BCs. Instead, we used neuronal makers such as the calcium-binding protein, parvalbumin, to label and identify the principal cell types, including BCs, in the mammalian cochlear nucleus (Pór et al., 2005).

Rhesus BCs immunostained robustly for parvalbumin, wherein the signal extended from the cell body to distal dendrites (Fig. 3F, 9, 11). We observed that, as in rodents, rhesus monkey BCs also formed clusters of 5 to 6 neurons. These clusters were observed more often in the rostral versus caudal sections of the AVCN. Within a cluster, BC dendrites oriented toward the cell body and dendrites of other identified BCs. (Figs. 9 and 11).

Divergent multiple-contact synapses within bushy cells

On the basis of parvalbumin immunostaining, the dendrites that surrounded the endings of BC cell bodies originated from other BCs (see above; Fig. 9). The ultrastructural characteristics of the dendritic profiles were similar to those in rodents (Gómez-Nieto and Rubio, 2009) and cats (Cant and Morest, 1979a; Tolbert et al., 1982; Rouiller and Ryugo, 1984; Ostapoff and Morest, 1991; Ryugo and Sento, 1991). Furthermore, we frequently observed primary dendrites emerging from BC somata that branched toward the cell body of a different BC (data not shown).

Our ultrastructural analysis showed that the MRnd and LRnd synapse types (Fig. 10), as well as the F and Pleo endings (data not shown), made divergent synaptic contacts on the cell body and the surrounding distal or proximal dendrites of identified GBCs and SBCs. Collectively, these results demonstrated that, as in rodents, higher nonhuman primates use a mechanism within the BC cluster to spread excitatory and inhibitory information.

Junctional connections within the bushy cell network

Dendrosomatic junctions—Proximal and distal dendrites and filopodia-like dendritic processes made contacts with BC somata (Fig. 11). Ultrastructurally, these dendrosomatic junctions were identified as puncta adherentia, plasma membrane subsurface cisternae, and

gap junctions whose average lengths ranged from $0.3 \pm 0.04 \mu\text{m}$ to $0.4 \pm 0.04 \mu\text{m}$. Plasma membrane subsurface cisternae were accompanied closely by an array of complex endoplasmic reticulum and formed close appositions that were indicative of gap junctions.

Dendrosomatic gap junction-like specializations were observed as sections of two adjacent plasma membranes, separated by a uniform gap of approximately 3–4 nm. Near these specializations lay membranes of the endoplasmic reticulum and large mitochondria, especially in the dendrite (Fig. 12). Divergent synaptic contacts (synaptic dyads) from excitatory and inhibitory endings that synapsed onto the cell body were also observed near the specialized junctions.

Dendrodendritic membrane appositions—Dendrodendritic membrane appositions between dendrites that were identified as BCs (data not shown), but these membranes did not bear any specialized junctions. Near these dendrodendritic appositions were endoplasmic reticulum, multivesicular bodies, and large clumps of mitochondria at either side of the junction. Similar to dendrosomatic junctions, the dendrodendritic membrane appositions were found near synaptic dyads.

Somasomatic junctions—By electron microscopy, we frequently observed two BC somata whose plasma membranes were juxtaposed and separated by 16–20 nm of extracellular space. We also noted regions where uniform gaps, 3–4 nm wide and $0.3 \mu\text{m}$ long, were present. These uniform and narrow gaps were formed by plasma membranes that were darker and thicker than the rest of the membrane and resembled gap junctions (Fig. 12C–F).

In addition, we also observed astrocytic profiles that surrounded the BC somata whose processes made contact with the plasma membrane of the neuron. In some cases, the inner face of the BC plasma membrane bore a complex of subsurface cisternae and formed a close apposition suggestive of a gap junction (Fig. 13). These cisternae appeared to be elongated plates ($0.3\text{--}0.4 \mu\text{m}$ in length) that were specifically restricted to the part of the BC soma adjacent to an astrocytic profile, or a part of the BC dendrite that abutted a BC soma. The junctional subsurface cisterna was often in close proximity to the endoplasmic reticulum.

These data suggest the existence of a BC network in the primate AVCN. Specialized membrane junctions are present between cells. Moreover, a divergence of synaptic connections suggest that afferent information is spread within a cluster in a BC network.

DISCUSSION

In this study, we report that the BCs in the rhesus monkey organize into neuronal clusters within which BCs form neuron-neuron connections and accept divergent multiple synaptic contacts (synaptic dyads: divergent synaptic inputs in which a single excitatory ending contact two different BCs) from incoming inputs. This is based on a light and electron microscopic analysis of the BCs soma and dendrites. We provide a detailed description of the fine structure and adjacent neuropil of BCs, as well as their neurotransmitter receptor types and synaptic inputs. Collectively, our data provide evidence of a similar synaptic organization for the BCs in primate and other mammals.

Globular and spherical BCs in the rhesus monkey anteroventral cochlear nucleus

Based on the size and microscopic appearance of the cell body and ultrastructure, two main types of BCs have been described in rodents and cats (Cant and Morest, 1979a, b; Gómez-Nieto and Rubio, 2009). Cells with similar morphological characteristics to BCs were found at the light microscopic level in higher primates and humans (Heiman-Patterson and

Strominger, 1985). Using similar criteria, we noted primarily two BC types in the AVCN of rhesus monkey. The distribution of rhesus BCs within the nuclear complex was similar to that in rodents (Mahendrasingam et al., 2004; Gómez-Nieto and Rubio, 2009) and cats (Cant and Morest, 1979a, b; Sento and Ryugo, 1989; Spirou et al., 2005). It was also similar to the one described for ovoid/spherical and globular cells in cats, higher primates, and humans (Osen, 1969; Moore and Osen, 1979; Heiman-Patterson and Strominger, 1985).

BC types are typically concentrated in separate regions within the AVCN (reviewed in Cant and Benson, 2003). In rodents (Wang et al., 1998; Cant, 1981; Mahendrasingam et al., 2004; Asako et al., 2005; Gómez-Nieto and Rubio, 2009) and cats (Cant and Morest, 1979a, b; Tolbert et al., 1982; Sento and Ryugo, 1989), the rostral region contains uniform, closely packed spherical BCs. In the rhesus AVCN, we observed more spherical neurons toward the rostral areas.

BC dendrites in rhesus also share ultrastructural features and branching patterns with those in cats (Ostapoff and Morest, 1991; Ryugo and Sento, 1991) and rats (Gómez-Nieto and Rubio, 2009). As in these species, the distal tufted dendrites distributed themselves and surrounded nearby cell bodies within the core. This organization of BC dendrites could regulate the interactions of BCs with neighboring cells and incoming inputs (Gómez-nieto and Rubio, 2009).

All BC types are defined by the axosomatic synapse of the endbulb, the specialized, large, complex auditory nerve terminal in the AVCN. Considering the variability between endbulbs (Nicol and Walmsley, 2002), there were no significant ultrastructural differences between those in the rhesus monkey and other nonprimate mammals (Nicol and Walmsley, 2002; Spirou et al., 2005; Gómez-Nieto and Rubio, 2009). This finding suggests that the physiological properties attributed to the specialized auditory inputs also exist in the endbulb-BC connection in rhesus monkeys.

As reported in rats (Gómez-Nieto and Rubio, 2009), the proximal and distal BC dendrites in rhesus receive synaptic contacts from terminals of the auditory nerve. In summary, the 2 types of BCs in the primate AVCN appear to be homologous to those in other mammals.

Synaptic integration in rhesus monkey BCs

The sound-evoked action potential of the BC results from the integration of acoustically driven excitatory and inhibitory inputs (Kopp-Scheinflug et al., 2002). The BC somata 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). In the present study, we distinguished at least 3 types of excitatory and 2 types of inhibitory terminals juxtaposed to the cell body and dendrites of BCs. More subtypes are possible, and they might be identified under different fixation conditions (Valdivia, 1970; Rubio et. al, 2008).

One of the excitatory terminals was identified as the endbulb from the cochlea. The endbulb uses glutamate to mediate rapid synaptic transmission through the activation of AMPA glutamate receptor complexes (Wang et al., 1998; Gardner et al., 2001; Whiting et al., 2009). As in rodents, the rhesus monkey AVCN and the BCs, in particular, express GluR3 and GluR4 AMPA receptor subunits and low levels of GluR2. These results suggest that the primate has a fast auditory nerve-BC synapse in which the AMPA receptor channel is Ca⁺⁺-permeable. Rhesus BCs also expressed the NMDA-NR1 and the delta1/2 glutamate receptor subunits. Recently, NMDARs in the mouse AVCN were reported to support transmission of temporal information at the endbulb (Pliss et al., 2009). In contrast, the function of delta glutamate receptors in the cochlear nucleus is unknown. Data on the adult cerebellum

indicate that this receptor type regulates synaptic plasticity (Yuzaki, 2003; Hirai et al., 2003). Whether similar functions can be attributed to the auditory nerve synapse is unknown. The auditory nerve synapse on fusiform cells of the dorsal cochlear nucleus shows no indication of synaptic plasticity (Fujino and Oertel, 2003). Thus, it is possible that the endbulb synapse also lacks synaptic plasticity. Therefore, the role of delta receptor subunits at this synapse raises issues that require further examination.

The second type of excitatory endings on rhesus BCs was larger than the MRnd endings and contained larger, round synaptic vesicles (LRnd). These endings share ultrastructural characteristics with mossy-like endings in the rat VCN (Zhang and Ryugo, 2007; Gómez-Nieto and Rubio, 2009), the fusiform cell layer in rats, the rhesus monkey dorsal cochlear nucleus (Rubio and Juiz, 1998; 2004; Rubio et al., 2008), and the rodent cerebellar granular cell layer (Xu-Friedman and Regehr, 2003). Our analysis demonstrated that rhesus BC somata and dendrites receive many VGLUT1 and VGLUT2 endings within the core. This finding is noteworthy, because VGLUT1 and VGLUT2 are associated with the cochlear and somatosensory pathways to the VCN, respectively (Zhou et al., 2007; Gómez-Nieto and Rubio, 2009). These data suggest that, as in rats (Gómez-Nieto and Rubio, 2009), rhesus BCs integrate excitatory cochlear and noncochlear information. New studies support this model and show that guinea pig BCs respond to trigeminal nuclear stimulation (Pradhan et al., 2010). A phylogenetic decrease in the granule cell population has been described in humans and higher nonhuman primates (Moskowitz, 1969; Moore, 1980; Moore and Osen, 1979; Rubio et al., 2008). The granular cell domain surrounds the core of the VCN and receives somatosensory inputs (Zhang and Ryugo, 2007; Zhou et al., 2007). Therefore, the existence of VGLUT2-positive mossy-like endings on rhesus BCs within the core raises the question of whether this synaptic input undergoes changes in higher primates and is distributed more internally within the nucleus.

The third type of synaptic ending (SRnd) was the smallest with regard to its size and the size and area of the synaptic vesicles. Ultrastructurally, these endings resemble cholinergic endings in the rodent cochlear nucleus and suggest that they contribute to the modulation of BC output (Benson et al., 1996; Gómez-Nieto et al., 2008a).

Inhibition of BCs also affects the processing of the spectral, temporal, and intensity cues of acoustic signals (Kopp-Scheinflug et al., 2002). AVCN neurons are sensitive to GABA and glycine (Wu and Oertel, 1986; Caspary et al., 1994; Ebert and Ostwald, 1995). As in rodents (Gómez-Nieto and Rubio, 2009), we found that different types of inhibitory synaptic endings exist on BC somata and dendrites. These endings contained flat or pleomorphic synaptic vesicles and made symmetrical synaptic contacts; thus, they can be considered glycinergic and/or GABAergic, (Altschuler et al., 1986; Luján et al., 2008) respectively. Supporting the model of mediation of inhibitory transmission in BCs, we observed expression of the GlyR α 1 subunit along the dendrites and cell bodies of BCs. In summary, the convergence of cochlear and noncochlear inputs into the cell body and dendrites influences the baseline activity in single BCs.

The bushy cell network is conserved between mammals

The present study provides evidence of a BC network in the rhesus monkey. We recently reported the existence of a BC network in the rat VCN (Gómez-Nieto and Rubio, 2009). Similar to the rat, the neuronal network of the rhesus is determined by divergent multiple synaptic contacts of the incoming synaptic inputs and direct neuron-neuron connections within BC clusters. In our material, BC dendrites oriented toward nearby BCs, and their distal dendritic processes formed nest-like clusters with neighboring BCs within the core. A similar dendritic organization has been reported in cats (Ryugo and Sento, 1991; Ostapoff and Morest, 1991) and rats (Gómez-Nieto and Rubio, 2009). As in rats, excitatory and

inhibitory synaptic inputs in primates established axodendro-somatic synaptic dyads between BCs. This finding suggests a general mechanism through which information is spread within the BC cluster. We also observed BCs that were connected to other putative BCs through dendrosomatic, dendrodendritic, and somasomatic membrane specializations. If functional, these membrane junctions might maintain cell-cell adhesion and provide electronic coupling between BCs within the cluster. Further studies are needed to determine the role of such a neuronal network in auditory processing.

Acknowledgments

We gratefully acknowledge Dr. Andrew Moiseff for comments and critically reading a previous version of the manuscript. We are thankful to Dr. Douglas Oliver for his comments, time and help editing the manuscript. We thank Drs. David Ryugo and Yolanda Smith for kindly providing the monkey tissue. The authors thank NIH/NIDCD RO1 DC006881 and NSF DBI- 0420580 for funds to purchase the Tecnai G2 Spirit Biotwin electron microscope.

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Figure 1. Globular bushy cells (GBC) in the caudal AVCN of the rhesus monkey

A, Micrograph shows a coronal section containing the caudal region of the AVCN where GBCs are preferentially located. **B**, Electron micrograph from the AVCN in (A) shows a typical GBC cell body and adjacent neuropil. In this ultrathin section nearly the entire cell body (CB) is contacted by identified auditory nerve endings (T1, colored in blue), and endings identified as inhibitory (T2–T3, colored in green). The inset shows detail of the rough endoplasmic reticulum. The GBC soma and the synaptic endings are surrounded by myelinated axons of different diameters (Ax), adjacent dendrites (D, colored in red), glial cells (gl) and blood vessels (bv). **T1**, High magnification electron micrographs show detail of the synaptic contacts of the auditory nerve on the GBC soma. **T2–T3**, High magnification electron micrographs show detail of inhibitory synaptic endings on GBC soma. High magnification of the postsynaptic densities (arrow) and synaptic vesicles are shown in the lower part of each panel. Scale bars: A, 500 μm; B, 10 μm; inset, 500 nm; T1–T2, 500 nm; High magnification: 125 nm.

Figure 2. Spherical bushy cells (SBC) in the rostral AVCN of the rhesus monkey

A, Micrograph shows a coronal section containing the rostral region of the AVCN where SBCs are preferentially located. **B**, Electron micrograph from the AVCN in (A) shows a typical SBC cell body and adjacent neuropil. The electron micrograph shows the entire surface of the SBC soma contacted by auditory nerve terminals (colored in blue), and inhibitory terminals (colored in green). The SBC soma and the synaptic endings are surrounded by myelinated axons of different diameters and adjacent dendrites (D, colored in red). Characteristic of SBC is the existence of rough endoplasmic reticulum that can be found concentrically located within the cytoplasm and around the nucleus (colored in purple). **C**, Electron micrograph of another SBC with the typical distribution of stacks (*) of the ER (colored in purple) within the cytoplasm. **D**, High magnification of the ER stacks highlighted in C with an *. Scale bars: A, 500 μm ; B, 5 μm ; C, 4 μm ; D, 500 nm.

Figure 3. Classification of Bushy cells (BCs) according to the cell body size and dendritic ultrastructure

A–B, Electron micrographs show a spherical bushy cell (SBC), and a globular bushy cell (GBC). Notice the differences in size of the cell body (CB) and the position of the nucleus (n). **C–D**, Histograms show the mean area ($\mu\text{m}^2 \pm \text{SD}$), and major diameter ($\mu\text{m} \pm \text{SD}$) of the cell bodies for the two cell types. Asterisks indicate a significant difference ($n = 30$). **E**, Micrographs show BCs immunopositive for parvalbumin. One or two dendritic trees (arrow-heads) emerged from the cell body. **F–H**, Electron micrographs show distal dendrites of putative SBCs in the rostral AVCN. These dendrites are characterized by large mitochondria, multivesicular bodies (mvb) and abundant membranes of smooth endoplasmic reticulum (ER). Scale bars: A–B, 10 μm ; E, 20 μm ; F–H, 500 nm, inset: 200 nm.

Figure 4. Fine structure of endbulb auditory nerve terminals on Bushy Cells (BCs) soma of the rhesus monkey AVCN

A, Low magnification electron micrograph of a globular bushy cell (GBC) in the AVCN. **B**, Electron micrograph of the boxed area in **A** shows a large terminal identified as arising from the auditory nerve. The auditory nerve terminal (t) contains medium-size clear round synaptic vesicles, and forms multiple asymmetric synaptic contacts on the cell body (inset from the asterisk-arrow in **E**). The synaptic contacts (arrows) form the typical convexities that characterize the synapse of the endbulb on GBC soma. The inset shows a detail of one of the PSDs (asterisk-arrow). Heminodes on axons that innervate BCs are recognizable by a series of paranodal loops (pairs of arrows). The heminode is typically located where the axon (Ax) enlarges into the nerve terminal (t). Asterisk indicates extended extracellular spaces filled with cellular processes. gl, glial cell; n, nucleus. Scale bars: **A**: 5 μ m; **B**, 500 nm.

Figure 5. Synaptic endings on dendrites of Bushy Cells (BCs)

A, Low magnification electron micrograph shows a longitudinal section of a primary dendrite (D) emerging from a SBC soma (CB). **B–F**, High magnification electron micrographs show detail of five synaptic endings on the dendrite. **B–D**, Details of the excitatory endings containing round synaptic vesicles and with asymmetric synaptic contacts on the dendrite (T1: colored in blue, T2: colored in pink, and T3: colored in red). **E–F**, Electron micrographs show detail of inhibitory terminals (colored in green) with pleomorphic (T4) and flat (T5) vesicles, and symmetric postsynaptic densities (arrows). Mit, mitochondria; mvb, multi-vesicular bodies; mt, microtubules. Scale bars: A, 2 µm; B–F, 500 nm; Insets: 200 nm.

Figure 6. Synaptic endings types contacting bushy cells (BCs) and classification of the synaptic vesicles

A, High magnification electron micrographs show details of the different synaptic vesicle types found in the excitatory and inhibitory synaptic terminals on BCs. Excitatory endings with large round (LRnd), medium round (MRnd), and small round (SRnd) synaptic vesicles; and inhibitory endings containing pleomorphic (Pleo) and flat (F) synaptic vesicles. Scale bar: A, 75 nm. **B**, Plot of the average roundness (ratio of minor to major ellipse diameters) versus the area (nm² ± SD) of synaptic vesicles of the five types of terminals on BCs (n = 30 synaptic vesicles per ending; Total endings = 47). **C**, Histograms show a comparison between the synaptic endings containing large (LRnd) and medium (MRnd) round synaptic vesicles on the cell body and on primary dendrites of BCs. Error bars represent SD. Asterisk indicates significant differences.

Figure 7. Neurotransmitter receptor types in bushy cells (BCs)

A, Micrograph shows a representative example of a coronal section through the anteroventral cochlear nucleus (AVCN). **B–E**, High magnification micrographs show BCs somata and dendrites immunopositive for NMDA (NR1), AMPA (GluR 2/3, GluR4), and delta 1/2 glutamate receptor subunits. **F**, High magnification micrographs show BCs somata and dendrites immunopositive for the glycine receptor alpha 1 subunit (GlyRa1). The immunolabeling in E and F were intensified with nickel. Scale bars: A, 500 μ m; B–F, 50 μ m. CC, cerebellar cortex.

Figure 8. Bushy Cells (BCs) of the AVCN rhesus monkey are decorated with VGLUT1 and VGLUT2 puncta

A and C, Light micrographs show sagittal sections through the cochlear nuclei immunostained for VGLUT1 (A) and VGLUT2 (C). **A1–2 and C1–2**, High magnification micrographs of the areas boxed in A and C, show detail of VGLUT1 and VGLUT2 immunolabeled puncta (arrowheads) around cell bodies and dendrites of BCs. **B and D**, Micrographs show large (endbulb-like) and small *en passant* VGLUT1 (B) and VGLUT2 (D) immunopositive endings on the cell body and along dendrites of BCs. Asterisks indicate the most distal dendritic branches around unlabeled BC somas. Scale bars: A–C: 1 mm; A1–C2, 25 µm; B and D 15 µm. AN: auditory nerve; AVCN, anteroventral cochlear nucleus; DCN, dorsal cochlear nucleus; PVCN, posteroventral cochlear nucleus; CRN, cochlear nerve root.

Figure 9. Parvalbumin immunostaining show that Bushy Cells (BCs) form neuronal clusters in the rhesus monkey AVCN

A, Micrograph shows a coronal section through the brainstem containing the AVCN and immunostained for parvalbumin. **B–C**, High magnification micrographs show two different planes of focus from the boxed area in **A**. **D**, Merge of the two images (**B**+**C**) shows BCs grouped in neuronal clusters. **E**, Camera lucida drawing of the BCs cluster shown in **D**. Notice BCs oriented their dendrites towards adjacent BC somata, which are frequently found next to each other. Scale bars: **A**, 500 μ m; **B–E**, 25 μ m. AN, auditory nerve; CC, cerebellar cortex.

Figure 10. Divergent multiple synaptic contact within bushy cell (BC) clusters

A, Low magnification electron micrograph shows a cell body (CB, colored in yellow) of a BCs cluster in the AVCN. **B–F**, High magnification electron micrographs show mossy-like terminals (T1, T3, LRnd terminals) and auditory nerve terminals (T2, T4, MRnd terminals) synapsing on the cell body and distal dendrites of SBC (D, colored in red) - synaptic dyads (arrows) -. Scale bars: A, 5 μ m; D, 2 μ m; B-C-E-F, 500 nm. Mitochondria (mit).

Figure 11. Dendro-somatic junctions within the bushy cell (BC) network

A–D, Light microscopy micrographs show BC clusters immunopositive for parvalbumin. Micrographs in **A–B**, are taken at two planes of focus and show the orientation of BC dendrites towards nearby BC somata. **C**, Micrograph of the merge images (**A+B**) and camera-lucida drawing (**D**) shows the distal tufted dendrites from one BC (black) encircling an adjacent BC soma (gray; arrow), this second neuron orients the dendrites towards a third BCs (pale gray; arrowhead). **E**, Low magnification electron micrograph shows distal dendrites (**D**) of a BC and synaptic endings containing round synaptic vesicles (**T1–T2**) on a BC cell body (**CB**). **F**, Electron micrograph of the area dashed in **E** shows how the distal dendrite contacts through a dendro-somatic junction the cell body of other BC. The apposition membranes are darker and resemble gap-junctions (arrows). Inset shows detail of the region between the arrows. **G**, High magnification electron micrograph shows a distal dendrite of a putative SBC making a membrane junction (gap junction; arrows) with the cell body of a BC. gl: glial cell processes; mit: mitochondria; mvb: multiple vesicular body. Scale bars: **A–D**, 15 μ m; **E**, 500 nm; **G**, 200 nm; **F**, 100 nm.

Figure 12. Somato-somatic junctions within the bushy cells (BC) network

A, Light micrograph shows two BC cell bodies (CB) immunopositive for parvalbumin and in close apposition (arrow). **B**, Toluidine blue staining on a semithin section of a BC cluster (CB1, CB2 and CB3) shows the somata of two BCs next to each other (arrow). **C**, Low magnification electron micrograph of the two BCs in B (CB1 and CB2). **D**, Montage of the boxed area in C, shows the plasma membrane of the two BCs contacting each other. Along this membrane apposition specialized membrane junctions along this membrane are identified as gap junction (arrows) **E**, Electron micrograph of the boxed area in D shows detail of the membrane specialization that resemble a gap junction between the two BC somata. **F**, Another example of gap junction between the plasma membrane of two cell bodies of BCs. Scale bars: A–B, 25 µm; C, 10 µm; D, 500 nm; E–F, 200 nm.

Figure 13. Astrocytic-somata junctions within the bushy cells (BC) network

Electron micrographs show representative examples of specialized membrane junctions between astrocytic processes (asterisk) and BC somata (A–D). ER: endoplasmic reticulum; Pleo: synaptic terminal with pelomorphous vesicles; MRnd: synaptic terminal with medium round vesicles identified as the auditory nerve. Scale bars: A–B: 100 nm; C–D: 500 nm.

Table 1**Primary Antibodies Used**

Antigen	Host	Immunogen	Manufacturer	Dilution
Vesicular glutamate 1 transporter (VGLUT1)	Rabbit	Strep-Tag fusion protein containing amino acid residues 456–560 of rat VGLUT1	Synaptic Systems (Göttingen, Germany), rabbit polyclonal, #135 302	1:1,000
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	1:1,000
Vesicular glutamate 2 transporter (VGLUT2)	Mouse	Recombinant protein from rat VGLUT2	Chemicon (Temecula, CA), monoclonal, #MAB5504	1:1,500
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), monoclonal, #MAB1572 lot No. LV1505479	1:1,500
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., 1992).	1:1,000
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., 1992).	1:1,000
NR1 NMDA receptor subunit	Rabbit	Synthetic peptide (LQNQKDTVLPRRAIER EEGQLQLCSRHRRES; amino acids 909–938) corresponding to the C-terminus of the rat NR1 subunit	Chemicon (Temecula, CA); polyclonal, #AB1516	1:1000
Delta 1/2 glutamate receptor subunit	Rabbit	Synthetic peptide (QPTPTLGLNLGNDPDR GTSI) corresponding to the C-terminus 20 amino acids of the rat glutamate $\delta 2$ subunit	Polyclonal Ab T25, gift from Robert Wenthold (Mayat et al., 1997).	1:1,000
Glycine receptor alpha 1 subunit (GlyR $\alpha 1$)	Mouse	Purified GlyR $\alpha 1$ peptide from rat spinal cord, (epitope corresponding to the N-terminus 96–105 residues of the rat $\alpha 1$ -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 et al., 2000)	Monoclonal Ab 4a Alexis Biochemicals (San Diego, CA), #804-037-C100	1:1500