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Research Report

Neuronal connexin expression in the cochlear nucleus of big brown bats

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ABSTRACT

We present immunohistochemical data describing the presence and distribution of connexins, structural component of gap junctions, in the cochlear nuclei of adult big brown bats (*Eptesicus fuscus*). Echolocating big brown bats show microsecond scale echo-delay sensitivity that requires accurate synchronization of neuronal responses to the timing of echoes. Midbrain and auditory cortical neuronal response timing is similar to that observed in other non-echolocating mammals, suggesting that lower auditory processing nuclei may have specialized mechanisms for obtaining the required temporal hyperacuity. Our data shows that connexin 36, a gap junction protein specific to neurons, is most densely expressed in the bat's cochlear nuclear complex, the medullary region that receives and processes first-order afferents from the auditory nerve. Cx36 expression is absent in the cochlear nucleus of normal mice, which have high-frequency hearing sensitivity similar to big brown bats. Glial connexins, Cx26 and Cx43, expressed in astrocytes and several inner ear structures, are also found in the bat cochlear nucleus complex, associated with major fiber tracts in and around the cochlear nuclei. The extensive presence of neuronally-associated Cx36 in brainstem auditory structures of adult bats suggests a possible role for gap junctions in mediating echo-delay hyperacuity.

1. Introduction

Echolocating big brown bats (*Eptesicus fuscus*) emit wideband (20–100 kHz) biosonar sounds and perceive objects from echoes that return to their ears. Their biosonar sounds are frequency-modulated (FM), and auditory mechanisms of echolocation are based on frequency-by-frequency registration of the timing of the FM sweeps in broadcasts and echoes (Neuweiler, 2000; Simmons et al., 1996). In behavioral tests, they can detect changes in echo delay and phase that amount to a few microseconds or less (Simmons et al., 1990, 2003; Masters et al., 1997; Moss and Schnitzler, 1995). This echo-delay hyperacuity is quantitatively related to the bandwidth of echoes (Simmons et al., 2004), implying frequency-by-frequency registration of the timing of sounds with unusual precision. To achieve this hyperacuity, initial auditory processing requires extremely accurate synchronization between stimulus occurrence and neuronal responses. Brainstem neuronal responses show high temporal precision at the single-cell level (Haplea et al., 1994), and may capture timing information by response synchrony and then

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Abbreviations: CN, cochlear nuclear complex; Ce, cerebellum; AVCN, anteroventral cochlear nucleus; PVCN, posteroventral cochlear nucleus; DCN, dorsal cochlear nucleus; Cx36, Connexin 36

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pass it on to midbrain and forebrain centers that transform stimulus timing information into intrinsically slower neuronal codes (Neuweiler, 2000; Simmons et al., 1996; Wotton et al., 2004). Animals, such as electric fish, that depend on precise synchronization between sensory input and early neuronal processing, utilize gap junctions at early stages of processing to mediate stimulus timing (Heiligenberg, 1991; Moortgat et al., 2000). Gap junctions are not known to occur in mammalian brainstem auditory nuclei, but the temporal hyperacuity of big brown bats suggests that they might be present in this species.

Vertebrate gap junctions are formed by hexamer configurations of trans-membrane proteins called connexins. Connexin 36 (Cx36) expression is limited to neurons (Condorelli et al., 1998; Rash et al., 2000), whereas other connexins, including Cx26 and Cx43, but not Cx36 (Connors and Long, 2004), are present in astrocytes and oligodendrocytes. Gap junctions formed from connexins are widely distributed in both neurons and glia during early postnatal development of the mammalian nervous system (Leung et al., 2002). Neuronal Cx36 is expressed in diverse regions of both the developing and adult brains, although expression in adult brains is primarily observed in regions with networks of inhibitory interneurons (Deans et al., 2001; Condorelli et al., 2000). Glial expression of connexins remains robust in adults, and is associated with gap junctions both in the brain and in peripheral sensory structures, including the inner ear (Forge et al., 2003; Kihara et al., 2006; Jagger and Forge, 2006). In contrast, there is a marked decrease in the presence of Cx36-based neuronal gap junctions as development progresses (Heister et al., 2007). In adults, they are found in regions of the brain that are dependent on high fidelity synchronization of activity in local networks. Such regions include the retina (mouse, Han and Massey, 2005), the inferior olive (rat, Placantonakis et al., 2004; mouse, Weickert et al., 2005), the thalamic reticular nucleus (rat, mouse, Landisman et al., 2002; mouse, Liu and Jones, 2003), the CA1 and CA3 regions of the hippocampus (mouse, Weickert et al., 2005; Buhl et al., 2003; Parenti et al., 2000) and the somatosensory cortex (rat, Gibson et al., 1999). Knockout mutant mice that lack the Cx36 gene show diverse neural effects, ranging from increased asynchronous retinal action potentials (Torborg et al., 2005) to memory and motor impairment (Frisch et al., 2005).

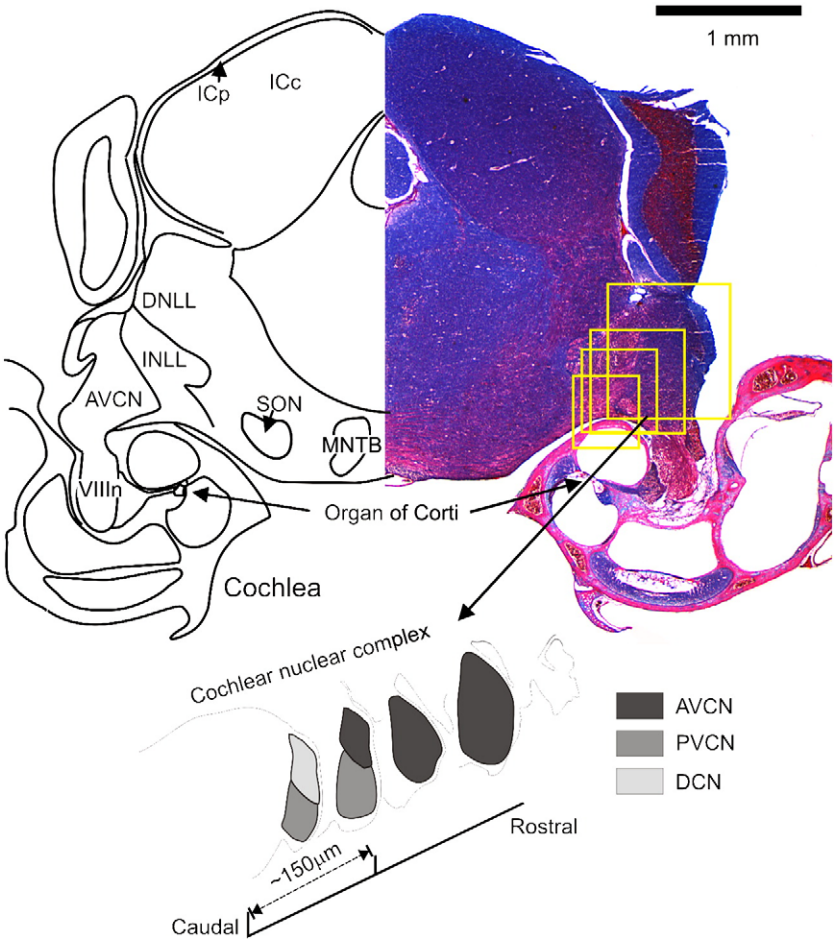

Fig. 1 – 5 μm thick coronal Gomori trichrome stained section through the bat brain shows major components of the peripheral and ascending auditory pathway (blue = neural cell bodies, red = fibers, purple = connective tissue and decalcified bone. Line images (bottom) show major components of the CN caudal to the trichrome section above. Yellow rectangles are indicators of approximate location of line drawings along an orthogonal caudal rostral line. DNLL, dorsal nucleus of the lateral lemniscus; ICc, central nucleus of the inferior colliculus; SON, superior olivary nucleus; MNTB, medial nucleus of the trapezoid body. Scale bar = 1 mm.

Given the big brown bat's need for more than ordinary temporal registration at early stages of echo processing, we hypothesize that gap-junction-based electrical synapses might play a role in interneuronal communication in the auditory brainstem. To explore this possibility, we examined the expression of connexins in the cochlear nuclear complex of the adult big brown bat and compared it to expression in the same region of normal mice, a species of similar size and similar high-frequency hearing sensitivity (Dalland, 1965; Heffner and Masterton, 1980; Koay et al., 1997). Any differences between their auditory systems can reasonably be attributed to the specialized requirements of echolocation rather than to ultrasonic hearing.

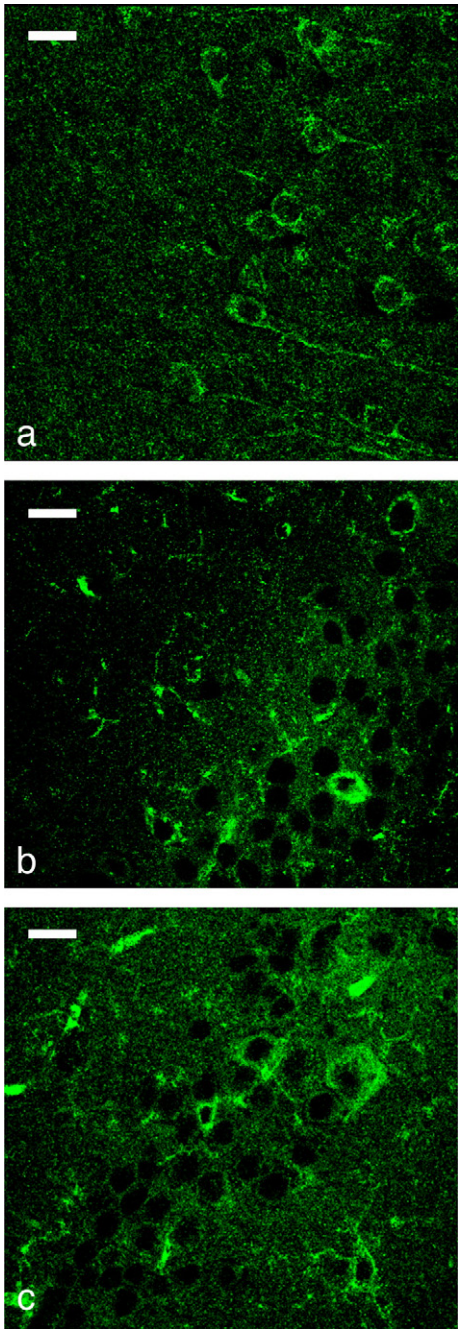

Fig. 2 – Confocal images of Cx36 labeled cells in the hippocampus of the bat and mouse. (a) Cx36 labeled interneurons in the CA3 region of the bat. (b) Cx36 labeled pyramidal cells in the CA1 region of the bat. (c) Cx36 labeled pyramidal cells in CA1 of the mouse. Dorsal is up, medial to the left, scale bar = 10 μm.

Table 1 – Relative density of connexin 36 protein in mouse (*N* = 3) and bat (*N* = 10)

Site	Bat	Mouse
Hippocampus	++	++
Reticular nucleus of the thalamus	+	+
Inferior olive	++	++
Auditory cortex	–	–
Inferior colliculus	–	–
Nucleus of the lateral lemniscus		
VnLL	–	–
InLL	–	–
DnLL	+ (3 of 10)	–
Cochlear nucleus		
PVCN	+++	–
DCN	+++	–
AVCN	++++	–
Cerebellum	++	++

Qualitative assessment of label: ++++ heavy, +++ moderate, ++ modest, + light, – none.

2. Results

2.1. *Structural anatomy*

The auditory periphery and brainstem of *Eptesicus* generally is similar to that of most mammals, but there are some specific organizational differences (Fig. 1). The basilar membrane of the organ of Corti is 12 mm long through its 2.5 turns. The cochlea lies close to the brainstem, so that the length of the auditory nerve (VIIIn) central processes from cell bodies of the spiral ganglia to the synaptic bed of the cochlear nuclear complex (CN) is only about 400–500 μm (Fig. 1). The CN is composed of three major subnuclei, the dorsal (DCN), posteroventral (PVCN), and anteroventral (AVCN) cochlear nuclei (Fig. 1), all of which receive first-order synaptic input from the VIIIn. Neuronal subclasses in each subnucleus project monaurally or binaurally to different sites in the ascending auditory pathway of the medulla and midbrain (Huffman and Covey, 1995; Covey, 2005).

2.2. *Connexin expression*

Connexin immunohistochemistry was carried out to identify four different subtypes of connexins in the CN in a total of 13 bat brains and 3 mouse brains: neuronal Cx36 (*N* bats = 10, mouse = 3), and glial connexins, Cx26 (*N* = 3), Cx32 (*N* = 9), and Cx43 (*N* = 2), all in bat brains. Controls included immunohistochemical procedures with omission of either primary and/or secondary antibodies.

2.3. *Cx36 expression*

Initial comparison of immunohistochemical label for neuronal Cx36 throughout the brain revealed similarities between the bat and the mouse. Comparing label in the hippocampus, similar

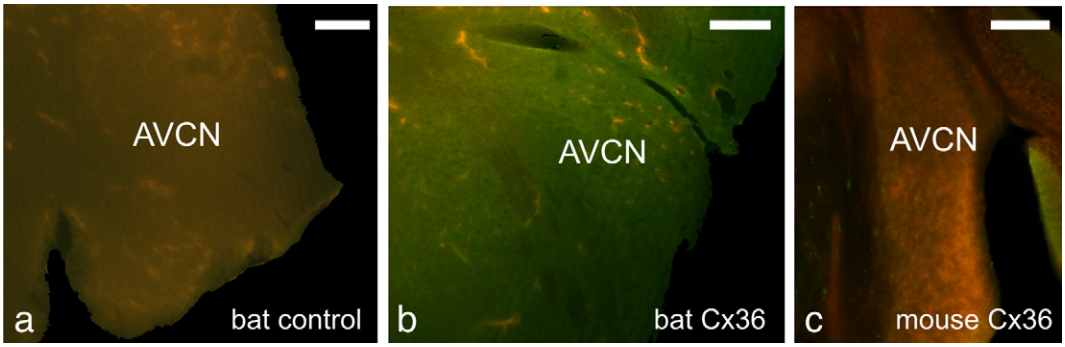

Fig. 3 – Distribution of Cx36 label in the CN. (a–c) Cx36 (green) expression in the AVCN using standard fluorescence optics. (a) No label in control section from bat with primary anti-Cx36 antibody omitted, (b), diffuse punctate Cx36 expression in serial section from same bat with antibody included, (c) lack of label in a mouse following same treatment as in experimental bat sections. Green structures along tubular elements in the mouse are perfusion artifacts and occasional astrocytic artifactual label, a phenomenon noted by other users of this clone in mouse (S. Patrick, personal communication). Green label in mouse cerebellar cell layers is Cx36 label, similar to that seen in the bat. Yellow–orange structures are perfusion artifacts. Scale bars (a–c) =200 μm.

Cx36 expression was observed in the CA3 and CA1 regions of the hippocampus (Fig. 2). There was qualitatively greater label in CA3 than in CA1 in both species. Cx36 label was identified primarily in multipolar, presumptive interneurons in CA3 (Fig. 2a), and in presumptive pyramidal cells in CA1 in bat and mouse (Figs. 2b,c), consistent with published findings in mouse and rat (Condorelli et al., 1998; Deans et al., 2001). We also found modest label in the inferior olivary region, the reticular thalamic nucleus, cellular layers of the cerebellum, and light label in the suprachiasmatic nucleus. Labeling was consistent in these regions between both species (Table 1). We did not note any Cx36 label in the auditory cortex in either the bat or the mouse.

The strongest and most consistent expression throughout the bat's brain was observed in regions dorsal to entrance point of the VIIIn in the CN (Fig. 3). Using conventional fluorescence optics, control sections (Fig. 3a, primary anti-Cx36

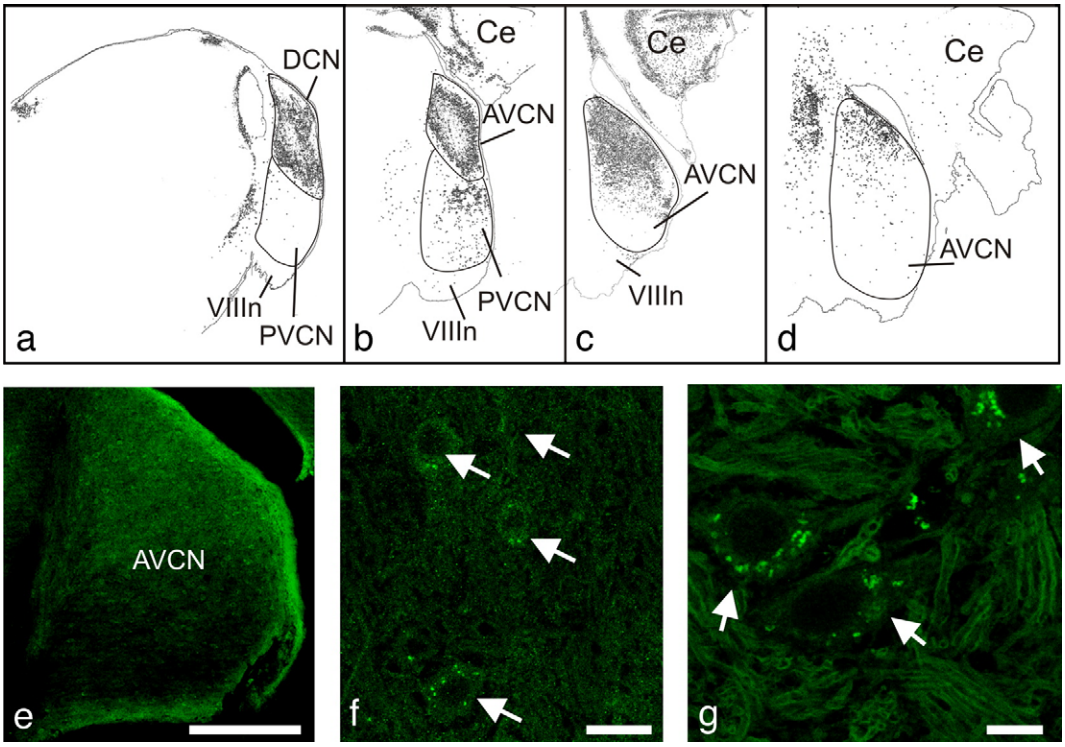

Fig. 4 – (a–d) Line drawings of Cx36 expression in sequential sections of the bat CN. Dots and enclosed granular areas represent regions of maximal Cx36 label. Puncta in the cerebellum (Ce) represent real Cx36 expression. (e–g) Confocal images of Cx36 labeled cells (green) in the CN of the bat. (e) Low power overview of Cx36 label in the AVCN. (f) Confocal image showing punctate Cx36 label of cells from the border of the DCN and AVCN. (g) Confocal image of cells in the DCN. Cell perikaryal regions with clear Cx36 label are marked with white arrows. Dorsal is up, medial is left in all images. Scale bars = (e) 100 μm, (f,g) = 10 μm.

antibody omitted) show no Cx36 label in the bat. No fluorescent label was observed in any control sections of bat or mouse tissue. In non-control sections, Cx36 expression in the bat AVCN shows diffuse label at low power (Fig. 3b), while a similar section from the mouse (Fig. 3c) shows no Cx36 label in the CN, although there is light label in the cerebellum similar to that observed in the bat (Table 1).

Generally, Cx36 expression in the bat was constrained to the dorsal regions of the cochlear nucleus across the entire rostrocaudal extent. Caudally, moderate labeling was found in the dorsal cochlear nucleus (DCN), near the most posterior region of the VIIIn at the border of the PVCN. Very little label was observed in the PVCN itself at this level (Fig. 4a). Rostral to the DCN, there was heavy label in the caudalmost AVCN, with some in the PVCN at this point (Fig. 4b). Further rostral, where the AVCN continues to the end of the CN, labeling remains moderately dense (Fig. 4c) until the CN reaches the level of the nucleus of the lateral lemniscus where label thins out substantially (Fig. 4d). While very light Cx36 labeling was sometimes found at the lateral margin of the inferior nucleus of the lateral lemniscus (INLL) (Figs. 1,4d, Table 1), this finding was not consistent in all bats (3 of 10).

Under confocal optics, low power images show that Cx36 is densely expressed in the dorsal region of the AVCN near the midpoint of its rostrocaudal extent (Fig. 4e). At higher power, puncta are observed in the perikaryal regions of cells in the AVCN. Sequential confocal images from a z-stack in the border region of the DCN and AVCN show Cx36 label of neural perikarya of similar looking round bodied cells (Fig. 4f,g). Qualitatively, approximately 10–20% of cells in any given image showed clear perikaryal labeling. This finding requires stereological analyses for confirmation; however, the proportion seemed consistent wherever there was moderate Cx36 label. Our findings do not allow us to absolutely identify specific populations of cells in this region based on their morphology, only that most of the label in this region seems to be perikaryal.

No consistent Cx36 expression was found in other elements of the ascending auditory pathway, including the inferior and dorsal nuclei of the lateral lemniscus, superior olivary nuclei, inferior colliculus and auditory cortex.

2.4. *Cx26, 32 and 43 expressions*

Cx26, Cx32, and Cx43 labeling has been observed in glia in adult mice and other mammals. These connexins were found in several areas of the bat brain, including fiber pathways of the ascending auditory system. Cx32 in particular was noted in the superficial peripheral nucleus of the inferior colliculus (ICp), as well as in periventricular regions of the IC and SC (data not shown). Glial connexins showed differential expression in the CN, with strong labeling by Cx26 and Cx43 and little or no labeling by Cx32 (Fig. 5). Cx26 (Fig. 5a) is expressed primarily in the lateral and medial AVCN, overlapping with the distribution of Cx36 (Figs. 4,5). Cx26 was also observed to label the tectospinal tract medial to the AVCN at this level. In addition, there was some bright labeling with limited distribution in the terminal region of the VIIIn. Cx32 (Fig. 5b) showed no significant distribution in this same region. Cx43 shows the widest distribution in this area (Fig. 5c), with moderate to heavy labeling in the synaptic-bed region of the AVCN, within the DCN and AVCN themselves, and strong labeling of tectospinal fibers and fiber bundles throughout the reticular gray and trigeminal sensory nucleus regions.

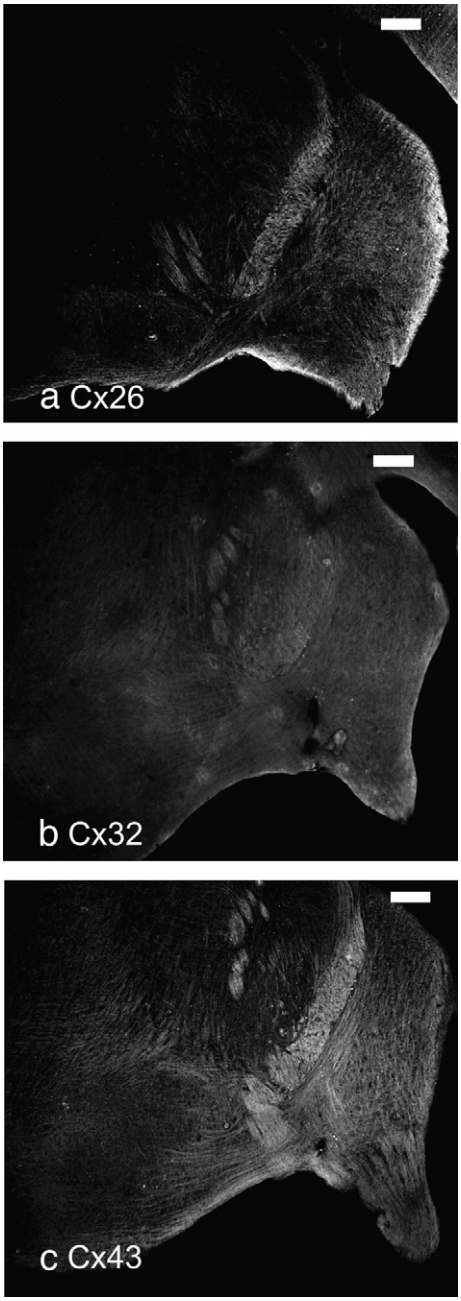

Fig. 5 – Glial-associated connexin expression in the region of the AVCN. (a) Cx26, (b), Cx32, and (c) Cx43. Light areas are regions of connexin expression. All three images have been contrast enhanced to allow visualization of margins in Cx32 which showed no significant label anywhere in the region. Dorsal is up, medial is left in all images. Scale bars = 200 μm.

3. Discussion

This study characterizes the distribution of neuronal- and glial-associated connexins in the central auditory system of

the echolocating big brown bat, with reference to the comparable distribution found in mice. Neuronal Cx36 shows similar distribution across non-auditory brain structures including the hippocampus, reticular thalamic nuclei, and inferior olive of both the big brown bat and the mouse. In the bat, however, Cx36 is heavily expressed in the cochlear nuclear complex, while it is absent in the mouse. While our findings did not identify specific cell type, confocal imaging generally showed perikaryal Cx36 label in round bodied neurons of approximately 20 mm diameter. Cx26 and Cx43, both of which are expressed in developing and mature inner ear structures (Forge et al., 2003) and in astrocytes in the brain (Nagy et al., 2001) were observed in the CN and surrounding non-auditory medullary fiber tracts. Cx32, which is associated specifically with oligodendrocytes (Connors and Long, 2004; Nagy et al., 2001), was observed in higher midbrain regions (inferior and superior colliculi) but showed no substantial or consistent expression in the CN. The dense expression of Cx36 in the CN suggests the existence of electrical gap junctions in the big brown bat's cochlear nuclear complex.

3.1. *Comparison of connexin expression*

Early *in situ* studies of CNS connexins described widespread distribution of Cx32 and Cx43 proteins throughout the brain (Micevych and Abelson, 1991). Initial study of the Cx36 gene based on rat and mouse brain and mouse retina showed high expression in the retina with age-dependent decrease in expression over development (Söhl et al., 1998). Subsequent studies demonstrated that Cx36 expression is limited to neurons (Condorelli et al., 1998) while connexins 26 and 43 are expressed in astrocytes and 32 in oligodendrocytes (Nagy et al., 2001). Northern blot and *in situ* hybridization studies that characterized neuronal Cx36 in the rodent brain demonstrated relatively widely distributed but discrete zones of expression (Condorelli et al., 1998). Expression of Cx36, Cx26, Cx32 and Cx43 in bats is largely similar to that seen previously in mice and rats, with the exception of the dense label of Cx36 in the cochlear nuclear complex, and the very limited expression of Cx32.

3.2. *Connexin expression in the auditory system*

Several connexin subtypes have a profound role in the function of the vertebrate auditory system. Cx26 and Cx30 are the primary structural components of homotypic and heterotypic gap junctions in the mammalian inner ear and are the only connexins expressed in the organ of Corti (Forge et al., 2003; Nickel et al., 2006; Zhao and Yu, 2006). Cx26 is highly expressed in the human cochlea and mutations in the gene have been implicated in hereditary sensorineural deafness (Kelsell et al., 1997). A single mutation (35delG) in the GJB2 gene that expresses Cx26 is one of the most common causes of heredity-linked hearing loss in European populations (Cohn and Kelley, 1999; Kelley et al., 2000). Functionally, Cx26-based gap junctions in the stria vascularis across development and in adult animals are implicated in maintaining the endocochlear potential (Forge et al., 1999). Cx43 has been identified in more limited regions of the mammalian inner ear, primarily in the vestibular sensory epithelium and in the lining of the semicircular canals, only appearing in the organ of Corti during development (Forge et al., 2003).

Despite the relatively widespread presence of gap junctions in the brain, there seems to be limited expression of connexin proteins in elements of the adult mammalian central auditory system. Cx32 and Cx43 were observed in the dorsal and ventral cochlear nuclei and inferior colliculus of the rat in early studies (Micevych and Abelson, 1991), but their expression is limited to oligodendrocytes and astrocytes, respectively. X-linked Charcot Marie Tooth syndrome is based on mutations of the gene that expresses Cx32 (Bergoffen et al., 1993) yielding CNS myelination deficits that can evoke a variety of sensory problems including auditory deficits, but this is more likely a secondary effect of slowing of CNS conduction velocity than a specific auditory pathology. While patch clamp data has implied the presence of electrical coupling in interneurons in layer I of the auditory cortex in young mice (Merriam et al., 2005), there has been no direct evidence for the presence of either gap junctions or Cx36 in this region in adult mice. The only direct role for gap-junction-based auditory function in the vertebrate CNS described to date is in Cx35 (the fish ortholog of mammalian Cx36) based gap-junction mediation of chemical synapses in Mauthner cells, elements of a circuit responsible for auditory-induced startle-like responses in fish (Pereda et al., 2003a, 2003b). This makes our findings of the dense expression of Cx36 in the bat cochlear nuclear complex all the more intriguing.

3.3. *Implications of Cx36 expression in the bat cochlear nucleus*

The presence of Cx36 in the big brown bat's auditory brainstem, as compared to the lack of expression in mice, indicates the possible existence of gap junctions between neurons in parts of the bat's CN, with potential consequences for early neuronal processing of auditory signals. Because individual brainstem and midbrain neurons in these bats typically produce only one spike per echolocation sound or echo (Simmons et al., 1996), rate-coding is absent at the single-cell level, leaving the bat's auditory system strictly dependent on accurate processing of the timing of spikes to generate perception of objects (Simmons et al., 1996; Ferragamo et al., 1998; Sanderson and Simmons, 2002; Haplea et al., 1994). Encoding of echo delay is likely based on frequency-by-frequency synchronization of neuronal responses between broadcasts and echoes across cells tuned to successive frequencies in the bat's FM signals. This requires synchronization of spikes between cells tuned to different frequencies early in the central auditory system. In addition, the occurrence of behavioral amplitude-latency trading specifically shows that bats detect changes of fractions of a microsecond in the timing of spikes across many neurons rather than detecting extraneous spectral cues (Simmons et al., 1990). The putative presence of interneuronal gap junctions in the bat cochlear nucleus suggests that this region may be a site for high speed synchronization of sensory input from the auditory nerve.

While the function of Cx36-mediated gap junctions in the adult mammalian brain is usually related to maintenance of timing in inhibitory networks, in the bat's cochlear nucleus, their function may be more analogous to the electrosensory system of electric fish. Behavioral tests have shown that electric

fish performing jamming-avoidance responses show temporal hyperacuity on the order of 1 μs (Heiligenberg, 1991), and that the electric organ discharge circuit is dependent on delay line circuits based on mixed chemical synapses and gap junctions (Moortgat et al., 2000). In the mormyrid electric fish, neurons in the command nucleus, medullary relay nucleus and bulbar command-associated nucleus are all electronically coupled within themselves and externally to generate electric organ discharges with timing accuracies on the order of 1 μs. (Bennett et al., 1967; Elekes and Szabo, 1985; Grant et al., 1986). Our current data does not allow us to determine if a similar coincidence/anticoincidence detector network exists within the cochlear nucleus of the big brown bat, and as such is a fertile ground for future research. Our data does suggest that the unique, dense expression of Cx36 in the bat cochlear nucleus may point to a role for electrical synapses at early stages of auditory processing in echolocation.

4. Experimental procedures

4.1. *Subjects*

Animal procedures used in these experiments are consistent with guidelines established by the National Institutes of Health and were approved by the Brown University Institutional Animal Care and Use Committee. Subjects were 13 wild-caught, big brown bats (8 females/5 males) housed in a colony room at Brown University on a 12:12 dark:light cycle with a controlled temperature of 22–25 °C. Bats were fed mealworms and provided with vitamin-enhanced water *ad libitum*. All subjects weighed between 14–19 g, were in good health and exhibited normal behavior. In addition, three wild type mice (*Mus musculus*) were included and treated using the same procedures for comparative purposes as this is the only mammalian species for which there are targeted Cx36 knockouts and thus extensive immunohistochemical data on connexin distribution.

4.2. *Immunohistology*

Bats and mice were killed by IP injection of 0.4 cm^3 Nembutal sodium (Abbott Laboratories, North Chicago, IL, USA) into the liver. Animals were transcardially perfused with 60 mL heparinized 0.9% saline followed by 60 mL 4% paraformaldehyde (pH 7.4). The brains were removed and postfixed for 24 h at 4 °C in 4% paraformaldehyde, then embedded in agarose (5% in 0.9% saline) and sliced by vibratomy (50 μm coronal sections).

Alternating brain sections were divided into multiple sets depending on the individual subject. In order to demarcate nuclear boundaries, each brain had a set for cresyl violet straining. This set was placed directly onto gelatin-subbed slides, delipidified with Citrisolv followed by xylenes (Fisher Scientific, Pittsburgh, PA), submerged in 0.5% (w/v) cresyl violet acetate solution (pH 3.7) and dehydrated using a graded ethanol series (70–100%). Slides were mounted using Cytoseal 60 (VWR, Bridgeport, NJ).

Each animal also had one, or several, sets for connexin immunohistochemistry: Cx36 (10 bats, 3 mice), Cx26 (3 bats) Cx32 (9 bats) and Cx43 (2 bats). Brain sections were suspended in conical tubes containing 900 μL of 1X PBT (pH 7.4), with 90 μL defined equine serum (HyClone, Logan, UT) and 10 μL primary antibody (Cx36: mouse monoclonal anticonnexin 36, Clone 1E5H5; Cx26: mouse monoclonal anticonnexin 26, clone CX12H10; Cx32 mouse monoclonal anticonnexin 32, clone CX2C2; Cx43 mouse monoclonal anticonnexin 43, clone CX-1B1; Zymed Laboratories, San Francisco, CA). These clones were derived from mouse and show reactivity with human tissue. Lack of data from bat-derived tissue prevents absolute characterization of sequence homology between bat and mouse. After 48 h shaking at 4 °C the sections were rinsed 3× with 20 mL 1X PBT and suspended in 20 mL 1X PBT with 2 mL defined equine serum (HyClone, Logan, UT) and 20 μL secondary antibody (goat α-mouse AlexaFluor 594 or goat α-mouse AlexaFluor 488, Invitrogen, Carlsbad, CA). After 24 h shaking at 4 °C the sections were rinsed 3× with 20 mL 1x100 PBT (pH 7.4) and mounted onto gelatin-subbed slides using AquaMount (Polysciences, Warrington, PA). Individual data sets were created by mounting consecutive sections from single brains on different gelatin-subbed slides to allow multiple histological treatments (Cx36, Cx26, Cx32, Cx43, cresyl violet, and controls with primary or secondary antibodies omitted).

One bat was killed and perfused as above for trichrome staining, the head removed and placed in decalcifying solution for 3 weeks (fixation decalcifying solution, Richard Allan Scientific, Kalamazoo, MI) with changes of solution every 5–7 days. Following decalcification, the head was stripped of external epidermis, embedded in paraffin, sectioned coronally at 5 μm and stained with Gomori trichrome. This allowed visualization of structures of the peripheral auditory system in relation to the ascending auditory system (Fig. 1). Identification of bat brain nuclei was carried out using published and unpublished atlases of *Eptesicus* and related microchiropterans (Baron et al., 1996, Covey and Casseday, personal communication).

4.3. *Confocal, fluorescent and brightfield imaging*

Sections were imaged using brightfield, fluorescent and confocal fluorescent techniques. Brightfield and fluorescent images were obtained using an Olympus BX60 microscope (Olympus, Melville, NY) equipped with custom made fluorescence cubes and an Optronics camera (Goleta, CA) connected to a Pentium 4 computer (Dell) running MagnaFire software on a Pentium 4 computer with Windows XP. Confocal imagery was taken using a Leica TCS SP2 MP confocal microscope (Leica TCS SP2, Leica Microsystems, Bannockburn IL). High power z-stack images were taken through each section and viewed in orthogonal planes using Leica software (Leica Microsystems). All images were saved as uncompressed 24 bit RGB TIFF files. General Cx36 distribution images were created by taking low power fluorescent images of half sections, brightness and contrast balancing the TIFFs, correcting for autofluorescence, wavelet high pass filtering the images and applying contour trace filters to generate outlines of labeled regions. Higher power standard fluorescence and confocal images were contrast and brightness adjusted to match relative brightness of labeled regions and remove

artifacts. Relative density of Cx label in experimental and control tissue in bats and mice was assessed by mean ratings by two authors familiar with anatomical regions on a scale from – (no label) to ++++ (heavy label) and reported in Table 1.

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