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CONNEXIN36 EXPRESSION IN MAJOR CENTERS OF THE AUDITORY SYSTEM IN THE CNS OF MOUSE AND RAT: EVIDENCE FOR NEURONS FORMING PURELY ELECTRICAL SYNAPSES AND MORPHOLOGICALLY MIXED SYNAPSES

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Abstract—Electrical synapses formed by gap junctions composed of connexin36 (Cx36) are widely distributed in the mammalian central nervous system (CNS). Here, we used immunofluorescence methods to document the expression of Cx36 in the cochlear nucleus and in various structures of the auditory pathway of rat and mouse. Labeling of Cx36 visualized exclusively as Cx36-puncta was densely distributed primarily on the somata and initial dendrites of neuronal populations in the ventral cochlear nucleus, and was abundant in superficial layers of the dorsal cochlear nucleus. Other auditory centers displaying Cx36-puncta included the medial nucleus of the trapezoid body (MNTB), regions surrounding the lateral superior olivary nucleus, the dorsal nucleus of the medial lemniscus, the nucleus sagulum, all subnuclei of the inferior colliculus, and the auditory cerebral cortex. In EGFP-Cx36 transgenic mice, EGFP reporter was detected in neurons located in each of auditory centers that harbored Cx36-puncta. In the ventral cochlear nuclei and the MNTB, many neuronal somata were heavily innervated by nerve terminals containing vesicular glutamate transporter-1 (vglut1) and Cx36 was frequently localized at these terminals. Cochlear ablation caused a near total depletion of vglut1-positive terminals in the ventral cochlear nuclei, with a commensurate loss of labeling for Cx36 around most neuronal somata, but preserved Cx36-puncta at somatic neuronal appositions. The results suggest that electrical synapses formed by Cx36-containing gap junctions occur in most of the widely distributed centers of the auditory system. Further, it appears that morphologically mixed chemical/electrical synapses formed by nerve terminals are abundant in the ventral cochlear nucleus, including those at endbulbs of Held formed by cochlear primary afferent fibers, and those at calyx of Held synapses on MNTB neurons.

Key words: Cochlear nucleus, Electrical synapses, Neuronal gap junctions, Connexin36, Mixed chemical/electrical synapses, Calyx of Held.

INTRODUCTION

Gap junctions at cell appositions are composed of connexin proteins that form intercellular channels allowing cell-to-cell passage of ions and small molecules (Goodenough and Paul, 2009; Herve and Derangeon, 2013). In neural systems, gap junctions between neurons create the structural substrate of electrical synaptic transmission (Bennett, 1997). The prevalence and physiological importance of electrical synapses between neurons in the CNS of lower vertebrates has long been recognized (Bennett and Goodenough, 1978; Sotelo and Korn, 1978). In contrast, it is only in the past decade that there has been general acceptance of the widespread occurrence and functional relevance of electrical synapses in neural circuitry of mammalian brain and spinal cord (Connors, 2009; Pereda et al., 2013; Pereda, 2014). The principal connexin component of neuronal gap junctions in mammalian systems is connexin36 (Cx36), which has been documented to occur in ultrastructurally identified gap junctions between neurons (Rash et al., 2000, 2001), and which supports electrical synaptic transmission in many regions of the CNS (Bennett and Zukin, 2004; Connors and Long, 2004; Hormuzdi et al., 2004; Söhl et al., 2005; Meier and Dermietzel, 2006; Bautista et al., 2012). Immunohistochemical visualization of Cx36 in gap junctions at the ultrastructural level is well-correlated with its localization by immunofluorescence, at least *in vivo* (Rash et al., 2004, 2005, 2007a,b), a fortuitous feature arising from what appears to be immunolabeling and detection of Cx36 exclusively at gap junctions, with its other potential subcellular and intracellular sites apparently remaining masked and undetectable with currently available anti-Cx36 antibodies (Nagy, 2012;

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Abbreviations: AVCN, anteroventral cochlear nucleus; CNIC, central nucleus of the inferior colliculus; CNS, central nervous system; Cx36, connexin36; DCIC, dorsal nucleus of the inferior colliculus; DCN, dorsal cochlear nucleus; DLL, dorsal nucleus of the lateral lemniscus; ECIC, external nucleus of the inferior colliculus; EGFP, enhanced green fluorescent protein; IC, inferior colliculus; LSO, lateral superior olivary complex; MNTB, medial nucleus of the trapazoid body; MSO, medial olivary nuclei; PBS, phosphate-buffered saline; PVCN, posteroventral cochlear nucleus; SOC, superior olivary complex; TBS, 50 mM Tris-HCl, pH 7.4, 1.5% NaCl; TBSTr, TBS containing 0.3% Triton X-100; vglut1, vesicular glutamate transporter-1.

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Nagy et al., 2013; Bautista and Nagy, 2014; Bautista et al., 2014). Thus, Cx36 represents at least one marker for allowing light microscopic immunofluorescence identification of Cx36-containing neuronal gap junctions and reveals their cellular localization. Notwithstanding current evidence for widely distributed networks of electrically coupled neurons, our ongoing surveys of Cx36 in the entire CNS of mice and rats continues to reveal numerous additional structures that display remarkable patterns of immunolabeling for Cx36 (Nagy, unpublished observations). These include brain regions that have not been previously explored for the existence of neuronal gap junctions, or those where evidence for electrical synapses has been reported, but which have not yet been examined by immunofluorescence labeling of Cx36 with the utility of this connexin providing a broadly defining marker of neuronal gap junctions. These structures are best exemplified by various nuclei in the auditory system, which are the focus of the present study.

The cochlear nuclei were among the first areas found to harbor neuronal gap junctions in the mammalian CNS. Within subdivisions of this structure, somato-somatic, somato-dendritic and dendro-dendritic gap junctions at what we refer to here as ''purely electrical synapses'' were reported to occur in the anteroventral cochlear nucleus (AVCN) as well as the dorsal cochlear nucleus (DCN) (Sotelo, 1975; Sotelo et al., 1976; Wouterlood et al., 1984; Mugnaini, 1985). In addition, another type of electrical synapse formed by gap junctions between an axon terminal and a postsynaptic neuron, which was found to have wide occurrence in lower vertebrates (Bennett and Goodenough, 1978) and termed ''mixed synapses'' with potential for dual chemical and electrical transmission, has been described in the ventral cochlear nucleus (Sotelo and Triller, 1982). Subsequent to these early reports, molecular and/or morphological correlates of electrical synapses, and electrical coupling via these synapses between cochlear neurons, has been the subject of only a few reports. As in most CNS regions found to contain neuronal gap junctions composed of Cx36, it has been noted that cochlear neurons express moderate levels of Cx36 mRNA (Condorelli et al., 2000). However, Cx36 protein was not detected in the cochlear nucleus of mouse, but was reported to occur in this nucleus of brown bats (Horowitz et al., 2008). More recently, ultrastructural studies have described neuronal gap junctions between the somata of bushy cells in the AVCN of rat and monkey (Gómez-Nieto and Rubio, 2009, 2011). In addition, *in vitro* electrophysiological approaches have demonstrated functional electrical coupling between stellate cells and fusiform cells in the dorsal cochlear nucleus (DCN) (Apostolides and Trussell, 2013).

Here, we examined Cx36 expression by immunofluorescence localization of this connexin in various subdivisions of the cochlear nucleus and other nuclei in the ascending auditory system in the CNS of mouse and rat. We used Cx36 knockout mice to confirm specificity of Cx36 detection, enhanced green fluorescent protein (EGFP)-Cx36 mice to correlate Cx36-promoter-driven EGFP expression with cellular distribution of Cx36, and cochlear ablation to establish primary afferent origin of the majority of immunolabeling for Cx36 in the ventral cochlear nuclei. A high level of Cx36 protein expression was found in association with neurons in each of the cochlear subnuclei, with distinct subcellular localization either at purely electrical synapses or at what appear to be morphologically mixed synapses in the ventral cochlear nuclei and in the MNTB, where Cx36 was co-localized with nerve terminals containing vesicular glutamate transporter-1 (vglut1). These results, together with moderately distributed immunolabeling of Cx36 observed in regions surrounding the lateral superior olivary nucleus, as well as within the dorsal nucleus of the medial lemniscus, all subnuclei of the inferior collicular, and in the auditory cerebral cortex, suggest that electrical synapses are a consistent feature in most divisions of the central auditory system.

EXPERIMENTAL PROCEDURES

Animals and antibodies

A total of twenty-two adult (> 2 months of age) male Sprague–Dawley rats, twenty adult male C57 BL/6-129SvEv mice and three CD1 adult male mice between two and three months of age were used in this study. Animals were obtained from the Central Animal Care Services at the University of Manitoba and utilized according to approved protocols by the Central Animal Care Committee of University of Manitoba, with minimization of the numbers of animals used. The C57 BL/6-129SvEv mice used included nine wild-type and three transgenic Cx36 knockout animals, colonies of which were established at the University of Manitoba through generous provision of wild-type and Cx36 knockout breeding pairs (Deans et al., 2001) from Dr. David Paul (Harvard). Eight transgenic adult male C57 BL/6-129SvEv mice included those in which Cx36 expression is normal and bacterial artificial chromosome provides EGFP expression driven by the Cx36 promoter, designated EGFP-Cx36 mice. These mice were taken from a colony established at the University of Manitoba starting with breeding pairs obtained from UC Davis Mutant Mouse Regional Resource Center (Davis, CA, USA; see also http://www.gensat.org/index.html). Data from mice and rats were compared for correspondence of results, and the mice further served to confirm antibody specificity of Cx36 detection by comparison of immunolabeling in wild-type *vs.* Cx36 knockout animals.

Two polyclonal antibodies (Cat. No. 36-4600 and Cat. No. 51-6300) and one monoclonal antibody (Cat. No. 39-4200) against Cx36 were obtained from Life Technologies Corporation (Grand Island, NY, USA) (formerly Invitrogen/Zymed Laboratories), and have been previously characterized for specificity of Cx36 detection in various regions of rodent brain (Li et al., 2004; Rash et al., 2007a,b; Curti et al., 2012). These antibodies were used at a concentration of 1–2 μg/ml in primary antibody incubations with tissue sections. Other antibodies included a guinea-pig polyclonal anti-vglut1 and a guinea-pig polyclonal anti-vglut2 obtained from Millipore