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MORPHOLOGICALLY MIXED CHEMICAL–ELECTRICAL SYNAPSES FORMED BY PRIMARY AFFERENTS IN RODENT VESTIBULAR NUCLEI AS REVEALED BY IMMUNOFLUORESCENCE DETECTION OF CONNEXIN36 AND VESICULAR GLUTAMATE TRANSPORTER-1

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Abstract—Axon terminals forming mixed chemical/electrical synapses in the lateral vestibular nucleus of rat were described over 40 years ago. Because gap junctions formed by connexins are the morphological correlate of electrical synapses, and with demonstrations of widespread expression of the gap junction protein connexin36 (Cx36) in neurons, we investigated the distribution and cellular localization of electrical synapses in the adult and developing rodent vestibular nuclear complex, using immunofluorescence detection of Cx36 as a marker for these synapses. In addition, we examined Cx36 localization in relation to that of the nerve terminal marker vesicular glutamate transporter-1 (vglut-1). An abundance of immunolabeling for Cx36 in the form of Cx36-puncta was found in each of the four major vestibular nuclei of adult rat and mouse. Immunolabeling was associated with somata and initial dendrites of medium and large neurons, and was absent in vestibular nuclei of Cx36 knockout mice. Cx36-puncta were seen either dispersed or aggregated into clusters on the surface of neurons, and were never found to occur intracellularly. Nearly all Cx36-puncta were localized to large nerve terminals immunolabeled for vglut-1. These terminals and their associated Cx36-puncta were substantially depleted after labyrinthectomy. Developmentally, labeling for Cx36 was already present in the vestibular nuclei at postnatal day 5, where it was only partially co-localized with vglut-1, and did not become fully associated with vglut-1-positive terminals until postnatal day 20–25. The results show that vglut-1-positive primary afferent nerve terminals form mixed synapses throughout the vestibular nuclear complex, that the gap junction component of these synapses contains Cx36, that multiple Cx36-containing gap junctions are associated with individual vglut-1 terminals and that the development of these mixed synapses is protracted over several postnatal weeks. **© 2013 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: electrical synapses, neuronal gap junctions, vesicular glutamate transporter-1.

INTRODUCTION

The long debate during the first half of the last century centering on whether inter-neuronal communication in the central nervous system (CNS) occurred by chemical or electrical means culminated around mid century with the demonstration of chemical transmission, followed shortly thereafter by the discovery of electrical transmission (reviewed in Bennett, 1977; Cowan and Kandel, 2001). The morphological substrate of (most) electrical synapses was identified to be neuronal gap junctions that form at close plasma membrane appositions and consist of connexin channel-forming proteins that allow intercellular movement of ions and small molecules (Bennett, 1997). While initially considered a less sophisticated form of communication widely utilized only in submammalian species (see however, Bennett, 2000), evidence slowly accumulated indicating electrical transmission between mammalian neurons in numerous CNS regions (see Nagy and Dermietzel, 2000). Nevertheless, it has been only in the past decade, with renewed interest in the area, that the importance and functional relevance of electrical transmission in mammalian CNS has become generally accepted (Bennett and Zukin, 2004; Connors and Long, 2004; Hormuzdi et al., 2004; Söhl et al., 2005; Meier and Dermietzel, 2006). Nearly all neuronal gap junctions so far identified in mammalian CNS occur at appositions between dendrites, somata or dendrites and somata. However, electrical communication can also occur between nerve terminals and postsynaptic elements; in fact, nerve terminals were among the first structures at which electrical transmission was found (Furshpan, 1964; reviewed in Bennett and Goodenough, 1978). Synaptic terminals with capabilities for electrical and chemical transmission, termed mixed synapses, have

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Abbreviations: CNS, Central nervous system; Cx36, connexin36; dLVN, dorsocaudal portion of the LVN; IgG, immunoglobulin G; ko, knockout; LVN, lateral vestibular nucleus; MVN, medial vestibular nucleus; MVNmc, medial vestibular nucleus magnocellular part; MVNpc, medial vestibular nucleus parvicellular part; SEM, standard error of mean; SpVN, spinal vestibular nucleus; SuVN, superior vestibular nucleus; TBSTr, TBS containing 0.3% Triton X-100; vglut-1, vesicular glutamate transporter-1.

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been extensively studied in lower vertebrates, among which the best characterized are the club endings on giant Mauthner cells in goldfish (Pereda et al., 2003, 2004). Morphological and/or electrophysiological evidence for morphologically mixed synapses has been reported in only a few locations in mammalian CNS, including the rat lateral vestibular nucleus (LVN) (Sotelo and Palay, 1970; Korn et al., 1973; Wylie, 1973; Sotelo, 1975; Sotelo and Korn, 1978; Sotelo and Triller, 1981), spinal cord (Rash et al., 1996) and hippocampus (Vivar et al., 2012; Hamzei-Sichani et al., 2012; Nagy, 2012).

In the LVN of rat, as well as mouse (cited as unpublished observations in Sotelo and Korn, 1978), gap junctions are formed between large nerve terminals and either the somata or dendrites of large neurons that themselves appear not to be directly coupled by interdendritic or soma-somatic gap junctions (Sotelo and Palay, 1970; Korn et al., 1973). Similar observations have been made in the vestibular nuclei or their anatomical equivalent in lower vertebrates, including lamprey (Stefanelli and Caravita, 1970), goldfish (Hinojosa, 1973), toadfish (Korn et al., 1977) and frog, (Sotelo, 1977) and in chick (Hinojosa and Robertson, 1967; Peusner, 1984). The terminals forming gap junctions in these species as well as in rat are either known or have been inferred to be of primary afferent origin based on demonstrations of electrical transmission by vestibular primary afferents in toadfish (Korn et al., 1977), frog (Precht et al., 1974; Babalian and Shapovalov, 1984), lizard (Richter et al., 1975), pigeon (Wilson and Wylie, 1970) and rat (Wylie, 1973). Nearly four decades after these early studies, mixed synapses in vestibular nuclei have received little attention, although glutamatergic transmission by primary afferents in these nuclei have been well studied (Highstein and Holstein, 2006). In the mammalian vestibular nuclear complex, there is no anatomical information available on the distribution and density of neuronal gap junctions, the connexin constituents of these junctions, or the source of terminals forming mixed synapses.

The family of connexin proteins that form gap junctional intercellular channels consists of twenty or twenty-one family members. The discovery that connexin36 (Cx36) is expressed in neurons (Condorelli et al., 1998; Söhl et al., 1998) has enabled histochemical approaches to demonstrate widespread neuronal expression of Cx36 in mammalian CNS, including Cx36 mRNA expression in neurons of rodent vestibular nuclei (Condorelli et al., 2000). In our studies of Cx36 protein expression in the brain and spinal cord, the anti-Cx36 antibodies used provide robust immunofluorescence labeling of Cx36 associated with neurons, and we have established the specificity of these antibodies in various CNS regions using Cx36 knockout (ko) mice (Li et al., 2008; Curti et al., 2012; Bautista et al., 2012). Immunolabeling of Cx36 occurs exclusively as immunopositive puncta, with no detection of intracellular labeling. We have confirmed that this punctate labeling corresponds to sites where we detect ultrastructurally identified neuronal gap junctions (Rash et al., 2000, 2001; Nagy et al., 2004; Rash et al., 2007a,b; Li et al., 2008). Further, there is a high correlation between presence of Cx36 and presence of functional electrical synapses (Bennett and Zukin, 2004; Connors and Long, 2004; Hormuzdi et al., 2004). While we cannot discount the possibility that additional connexins may be expressed in neurons, our studies have excluded neuronal localization of a variety of connexins expressed in the CNS (Rash et al., 2000, 2001, 2007a,b; Curti et al., 2012). Taken together, these points allow use of immunofluorescence labeling of Cx36 as the current best marker for electrical synapses in most regions of the mammalian CNS. Here, we describe abundant immunolabeling of Cx36 in vestibular nuclei of adult and developing rat and mouse, and report on the association of Cx36 with the nerve terminal marker vesicular glutamate transporter-1 (vglut-1). In addition, studies were undertaken to determine whether Cx36 association with vglut-1 in the vestibular nuclear complex reflects localization of Cx36 to terminals of primary afferent origin.

EXPERIMENTAL PROCEDURES

Animals and antibodies

Animals used in this study included a total of 44 adult Sprague–Dawley rats. For developmental studies, three rats were used at postnatal day 5, two at postnatal day 10, three at postnatal day 15, two at postnatal day 20, three at postnatal day 25 and the rest as adults. Included were fourteen adult rats taken for surgical procedures involving labyrinthectomy and vestibular nerve section. Mouse strains used included fourteen adult CD1 mice, three of these mice at postnatal day 5, and three at postnatal day 10 and the rest as adults. In addition, colonies of C57 BL/6-129SvEv wild-type and Cx36 ko mice were established at the University of Manitoba through generous provision of wild-type and Cx36 ko breeding pairs from Dr. David Paul (Harvard). Five adult wild-type and three adult ko mice were used from these colonies. Expression of Cx36 protein was investigated in the vestibular nuclei of both rat and mouse, because some early studies relevant to neuronal gap junctions in these nuclei were conducted in rat, and because examination of mouse provides the opportunity to confirm antibody specificity of Cx36 detection using wild-type *vs.* Cx36 ko mice. Studies were also conducted on brains harvested from three adult cats and three adult Hartley guinea pigs. Tissues from some of the animals were employed in parallel unrelated studies; therefore the numbers indicated do not reflect total cumulative usage in separate publications from our laboratory. Animals were utilized according to approved protocols by the Central Animal Care Committee of University of Manitoba, with minimization of the numbers of animals used.

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

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