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CONNEXIN36 IDENTIFIED AT MORPHOLOGICALLY MIXED CHEMICAL/ELECTRICAL SYNAPSES ON TRIGEMINAL MOTONEURONS AND AT PRIMARY AFFERENT TERMINALS ON SPINAL CORD NEURONS IN ADULT MOUSE AND RAT

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Abstract—Morphologically mixed chemical/electrical synapses at axon terminals, with the electrical component formed by gap junctions, is common in the CNS of lower vertebrates. In mammalian CNS, evidence for morphologically mixed synapses has been obtained in only a few locations. Here, we used immunofluorescence approaches to examine the localization of the neuronally expressed gap junction forming protein connexin36 (Cx36) in relation to the axon terminal marker vesicular glutamate transporter-1 (vglut1) in the spinal cord and the trigeminal motor nucleus (Mo5) of rat and mouse. In adult rodents, immunolabeling for Cx36 appeared exclusively as Cx36-puncta, and was widely distributed at all rostro-caudal levels in most spinal cord laminae and in the Mo5. A high proportion of Cx36-puncta was co-localized with vglut1, forming morphologically mixed synapses on motoneurons, in intermediate spinal cord lamina, and in regions of medial lamina VII, where vglut1-containing terminals associated with Cx36 converged on neurons adjacent to the central canal. Unilateral transection of lumbar dorsal roots reduced immunolabeling of both vglut1 and Cx36 in intermediate laminae and lamina IX. Further, vglut1-terminals displaying Cx36-puncta were contacted by terminals labeled for glutamic acid decarboxylase65, which is known to be contained in presynaptic terminals on large-diameter primary afferents. Developmentally, mixed synapses begin to emerge in the spinal cord only after the second to third postnatal week and thereafter increase to adult levels. Our findings demonstrate that axon terminals of primary afferent origin form morphologically mixed synapses containing Cx36 in broadly distributed areas of adult rodent spinal cord and Mo5.

Key words: gap junctions, vesicular glutamate transporter-1, motoneurons, interneurons.

INTRODUCTION

A surge of reports over the past decade on electrical synapses formed by gap junctions between neurons in mammalian brain was fueled in part by the discovery of the first gap junction forming protein expressed in neurons, namely, connexin (Cx36) (Condorelli et al., 1998; Söhl et al., 1998). Subsequent findings of widespread neuronal expression of Cx36 (Condorelli et al., 2000), demonstrations of Cx36 in ultrastructurally-identified neuronal gap junctions in adult rodent brain (Rash et al., 2000, 2001a,b, 2007a,b; Nagy et al., 2004), and indications of the physiological relevance of these junctions, partly deduced from functional deficits in Cx36 knockout (ko) mice, all contributed to the general acceptance of the prevalence and importance of electrical transmission in most major regions of mammalian CNS (Bennett and Zukin, 2004; Connors and Long, 2004; Hormuzdi et al., 2004; Söhl et al., 2005; Meier and Dermietzel, 2006). Specifically, electrical coupling is considered to generate synchrony of subthreshold membrane oscillations and to promote synchronous recruitment of rhythmic firing (Bennett and Zukin, 2004; Connors and Long, 2004), which is emerging as a key feature of information processing in neuronal networks (Singer, 1999; Whittington and Traub, 2003; Senkowski et al., 2008). Nearly all electrical synapses so far studied in mammalian brain occur between dendrites and/or somata of specific classes of neurons. However, gap junctions can also be found at axon terminals, as in many systems of lower vertebrates (Bennett and Goodenough, 1978; Bennett, 1997), creating ‘‘mixed synapses’’ that provide for dual chemical and electrical neurotransmission. Mixed synapses in rodent CNS have been described in only a few CNS areas, including the lateral vestibular nucleus (LVN) (Sotelo and Palay, 1970; Korn et al., 1973; Nagy et al., 2013), hippocampus (Hamzei-Sichani et al., 2012; Nagy, 2012; Vivar et al., 2012) and spinal cord (Rash et al., 1996).

Electrical synapses and neuronal gap junctions in mammalian spinal cord have received far less attention than those in brain. In developing and juvenile rodent spinal cord, there is gap junction-mediated coupling between distinct subsets of interneurons (Hinckley and

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Abbreviations: C, cervical; CeCv, central cervical nucleus; Cx36, connexin36; EM, electron microscopy; GAD65, glutamic acid decarboxylase65; ko, knockout; L, lumbar; LVN, lateral vestibular nucleus; Mo5, trigeminal motor nucleus; PD, postnatal day; T, thoracic; TBS, 50 mM Tris–HCl, pH 7.4, 1.5% NaCl; TBSTr, TBS containing 0.3% Triton X-100; vglut1, vesicular glutamate transporter-1; vglut2, vesicular glutamate transporter-2.

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Ziskind-Conhaim, 2006; Wilson et al., 2007; Bautista et al., 2012) as well as between motoneurons, with progressive loss of motoneuronal coupling during the second week of life (Fulton et al., 1980; Arasaki et al., 1984; Walton and Navarette, 1991; Bou-Flores and Berger, 2001). Electrical coupling between developing motoneurons was suggested to support synchronous neuronal activity prior to the maturation of chemical synaptic connectivity (Kiehn et al., 2000; Tresch and Kiehn, 2000, 2002; Kiehn and Tresch, 2002). Analysis of connexin mRNA and/or protein expression has suggested that multiple connexins are expressed in spinal and trigeminal motoneurons, including Cx26, Cx32, Cx36, Cx37, Cx40, Cx43 and Cx45 (Matsumoto et al., 1991, 1992; Micevych and Abelson, 1991; Chang et al., 1999, 2000; Chang and Balice-Gordon, 2000; Personius and Balice-Gordon, 2000, 2001; Personius et al., 2001, 2007), raising the possibility that each of these connexins could form functional gap junctions between motoneurons. However, our recent analysis of connexin expression among trigeminal and spinal cord motoneurons in postnatal and adult rodent, using antibodies specific for each connexin, found labeling of only Cx36 in these neurons (Bautista and Nagy, 2013; Bautista et al., 2013). Our results suggest that Cx36 is the major connexin mediating the electrical and dye-coupling observed between motoneurons at early postnatal ages.

In spinal cord as in brain, neuronal gap junctions and/or functional neuronal coupling persist among specific neuronal populations in adult animals (Matsumoto et al., 1988, 1989; Logan et al., 1996; van der Want et al., 1998; Coleman and Sengelaub, 2002). Recently, we reported the expression of Cx36 in ventral and intermediate regions of the lumbar spinal cord of adult mice, and the loss of sensory-evoked presynaptic inhibition in mice lacking Cx36 (Bautista et al., 2012). The present study was motivated in part by our detection of abundant labeling of Cx36 in most spinal cord laminae, including extensive immunolabeling for Cx36 among motoneurons in lamina IX in adult rat and mouse (Bautista et al., 2012, 2013; Bautista and Nagy, 2013). The persistence of Cx36-puncta in the adult spinal cord, particularly those associated with motoneurons, and the presence of ultrastructurally-identified gap junctions associated with some populations of motoneurons in adult rodent spinal cord (Matsumoto et al., 1988, 1989; Rash et al., 1996; van der Want et al., 1998) appears to be at odds with the developmental loss of electrical coupling and dye-coupling between motoneurons (Fulton et al., 1980; Walton and Navarette, 1991; Chang et al., 1999). To reconcile this disparity, the present study provides a more comprehensive analysis of the immunofluorescence localization of Cx36 in the ventral horn and deep dorsal horn laminae as well as the trigeminal motor nucleus (Mo5) of mouse and rat spinal cord. In addition, we considered subcellular locations at which Cx36-containing neuronal gap junctions occur. We investigated the association of Cx36-containing gap junctions with axon terminals; an association that would suggest the presence of mixed synapses capable of dual chemical-electrical transmission in the adult spinal cord. Specifically, we examined the localization of Cx36 in relation to a marker of excitatory axon terminals of myelinated lumbar sensory afferents, namely vesicular glutamate transporter-1 (vglut1; Alvarez et al., 2004), in relation to vesicular glutamate transporter-2 (vglut2), a marker of other excitatory intraspinal axon terminals (Persson et al., 2006) and in relation to a marker of axon terminals containing glutamic acid decarboxylase65 (GAD65) that are in part associated with inhibitory synapses on sensory afferents (Hughes et al., 2005).

EXPERIMENTAL PROCEDURES

Animals and antibodies

Animals used included 22 adult (30–50 days old) male Sprague–Dawley rats, and eighteen adult wild-type mice and four Cx36 ko mice from colonies of C57BL/6-129SvEv mice (Deans et al., 2001) that were established at the University of Manitoba through generous provision of breeding pairs of these mice from Dr. David Paul (Harvard). In addition, four male rats and four male wild-type mice were used at each of various developmental ages, including postnatal day (PD) 5, 10, 15, 20, 25 and 30. Tissues from some of these animals were taken for use in parallel unrelated studies. Animals were utilized according to approved protocols by the Central Animal Care Committee of University of Manitoba, with minimization of the numbers animals used.

Three different antibodies against Cx36 were obtained from Life Technologies Corporation (Grand Island, NY, USA) (formerly Invitrogen/Zymed Laboratories), and included two rabbit polyclonal antibodies (Cat. No. 36-4600 and 51-6300) and one mouse monoclonal antibody (Cat. No. 39-4200), each used in incubations of tissue sections at a concentration of 1–2 μg/ml. The monoclonal anti-Cx36 was used for most of the studies involving double or triple immunolabeling with two or three different primary antibodies. Specificity characteristics of Cx36 detection by the anti-Cx36 antibodies in various regions of the rodent brain have been previously reported (Li et al., 2004; Rash et al., 2007a,b; Curti et al., 2012). Additional antibodies included: guinea-pig polyclonal antibodies against vglut1 and vglut2 obtained from Millipore (Temecula, CA, USA), and used at a dilution of 1:1000; and an anti-GAD65 obtained from BD Biosciences (Birmingham, UK) and used at a dilution of 1:100. In addition, we used a chicken polyclonal anti-peripherin antibody (obtained from Millipore and diluted of 1:500) to identify motoneurons and primary afferent fibers in the spinal cord. Peripherin is an intermediate filament protein highly expressed in neurons of the peripheral nervous system, but is also found in central neurons that have projections to peripheral structures, including motoneurons which display intense labeling for this protein (Clarke et al., 2010). Various secondary antibodies included Cy3-conjugated goat or donkey anti-mouse and anti-rabbit IgG (Jackson ImmunoResearch