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Structural and Intermolecular Associations Between Connexin36 and Protein Components of the Adherens Junction–Neuronal Gap Junction Complex

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Abstract—Intimate structural and functional relationships between gap junctions and adherens junctions have been demonstrated in peripheral tissues, but have not been thoroughly examined in the central nervous system, where adherens junctions are often found in close proximity to neuronal gap junctions. Here, we used immunofluorescence approaches to document the localization of various protein components of adherens junctions in relation to those that we have previously reported to occur at electrical synapses formed by neuronal gap junctions composed of connexin36 (Cx36). The adherens junction constituents N-cadherin and nectin-1 were frequently found to localize near or overlap with Cx36-containing gap junctions in several brain regions examined. This was also true of the adherens junction-associated proteins α-catenin and β-catenin, as well as the proteins zonula occludens-1 and AF6 (aka, afadin) that were reported constituents of both adherens junctions and gap junctions. The deployment of the protein constituents of these junctions was especially striking at somatic contacts between primary afferent neurons in the mesencephalic trigeminal nucleus (MesV), where the structural components of adherens junctions appeared to be maintained in connexin36 null mice. These results support emerging views concerning the multi-molecular composition of electrical synapses and raise possibilities for various structural and functional protein–protein interactions at what now can be considered the adherens junction–neuronal gap junction complex. Further, the results point to intracellular signaling pathways that could potentially contribute to the assembly, maintenance and turnover of this complex, as well as to the dynamic nature of neuronal communication at electrical synapses.

Key words: electrical synapses, electrical coupling, cell–cell adhesion, intracellular signaling, mesencephalic trigeminal nucleus, immunofluorescence.

INTRODUCTION

Gap junctions are composed of connexins that form hexamers (connexons) in plasma membranes (Evans and Martin, 2002; Goodenough and Paul, 2009). Clustered connexons in apposing membranes dock to create pores that allow cell-to-cell passage of ions and small molecules. Among the family of twenty connexins, connexin36 (Cx36) is widely expressed in neurons of developing and adult central nervous system (CNS) (Condorelli et al., 2000; Nagy et al., 2018) and Cx36 has been localized to gap junctions between neurons (Rash et al., 2000, 2001, 2004, 2007a,b; Nagy et al., 2004), where it forms electrical synapses (Bennett, 1997). Work over the last two decades has indicated the prevalence and physiological relevance of electrical synapses formed by Cx36-containing neuronal gap junctions (nGJs) in most major regions of mammalian CNS, where these synapses contribute to neuronal network activity (Connors and Long, 2004; Connors, 2009; Pereda et al., 2013; Pereda, 2014). Also now clear is the dynamic nature of electrical synapses and the high degree to which nGJs are regulated, exhibiting plasticity of their synaptic strength (Haas et al., 2011; Haas and Landisman, 2012; Pereda, 2014; Mathy et al., 2014; Turecek et al., 2014). This regulation may occur not only by Cx36 phosphorylation as has been reported (Urschel et al., 2006; Kothmann et al., 2009; O'Brien, 2014), but very likely also by the interplay between a growing list of

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Abbreviations: AJ–nGJ, adherens junction–neuronal gap junction complex; CNS, central nervous system; Cx36, connexin36; E-cadherin, epithelial cadherin; MesV, mesencephalic trigeminal nucleus; MUPP1, multi-PDZ protein-1; N-cadherin, neural cadherin; nGJs, neuronal gap junctions; PDZ, PSD-95, DlgA, ZO-1 domains; SIM, structured illumination microscopy; TBS, 50 mM Tris–HCl containing 1.5% sodium chloride; TBST, 50 mM Tris–HCl containing 1.5% sodium chloride and 0.3% Triton X-100; ZO-1, zonula occludens-1.

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nGJ-associated proteins (Hervé et al., 2012) that constitute an electrical synapse (Lynn et al., 2012). This interplay includes interactions of these proteins with each other and with intracellular signaling pathways (Nagy et al., 2018). Identification of nGJ-associated proteins and their functions represents a major challenge in the field of electrical synapses, progress on which provides understanding of mechanisms whereby these synapses are regulated and clues toward possible mechanistic causes of neurological disorders involving malfunctions of nGJs arising from disruption of their associated structural or regulatory components.

With a focus on deciphering the molecular constituents of nGJs, we have previously reported the localization of several proteins at nGJ, including zonula occludens-1 (ZO-1), the effector protein AF6 (aka, afadin), the scaffolding protein multi-PDZ protein-1 (MUPP1), and the cytoskeleton interacting protein cingulin, and we have shown that Cx36 directly interacts with some of these proteins (Li et al., 2004a,b, 2008, 2009, 2012; Lynn et al., 2012). These interactions were found to be mediated by the carboxy (C)-terminus motif of Cx36, which serves as a ligand for interaction with PDZ (PSD-95, DlgA, ZO-1) domains contained in each of ZO-1, AF6 and MUPP1. Based on these findings, we drew attention to the emerging parallel between the protein compositions of Cx36-containng nGJs and other cell–cell junctions such as adherens junctions and tight junctions (Lynn et al., 2012). Beyond these molecular similarities, functional relationships have been established between gap junctions and adherens junctions in peripheral tissues (Derangeon et al., 2009), with reports demonstrating that cell adhesion mediated via neural cadherin (N-cadherin) or epithelial cadherin (E-cadherin) is required for the formation of gap junctions (Keane et al., 1988), including those composed of connexin40 and connexin43 (Meyer et al., 1992; Hertig et al., 1996; Segretain and Falk, 2004; Li et al., 2005). In the CNS, nGJs have been frequently found in ultrastructural studies to be either in continuity or nearly so with adherens junctions in a variety of brain regions (Gwyn et al., 1977; Sloper and Powell, 1978; Kosaka, 1983; Katsumaru et al., 1988; Peters et al., 1991; Kosaka and Kosaka, 2003), but inter-relationships of the molecular constituents of these two types of junctions between neurons has not been examined.

Consideration of mechanisms underlying possible structural interactions between adherens junctions and nGJs requires understanding of the arrangement of some of the proteins associated with each of these junctions. The core proteins of adherens junctions are cadherins and nectins, which have domains that extend outside of plasma membranes, span the extracellular space, and interact homophilically to form cell–cell adhesions. It is known that nectins interact with another component of adherens junctions, namely AF6, that cadherins bind the additional components α-catenin and β-catenin, and that the nectin–AF6 and cadherin–catenin systems associate through AF6–α-catenin interaction (Shapiro and Weis, 2009; Harris and Tepass, 2010) to co-operatively organize these junctions (Miyahara et al., 2000; Takai and Nakanishi, 2003; Mori et al., 2014). The association of ZO-1 and AF6 with protein components of both adherens junctions and nGJs (Itoh et al., 1993; Mandai et al., 1997; Takahashi et al., 1999; Li et al., 2004a, 2012), together with the known interaction partners of these two proteins, raise possibilities for molecular links between these two types of intercellular junctions. Here, we used immunofluorescence approaches to examine the neuronal plasma membrane localization of various proteins components of nGJs and adherens junctions in various brain regions of adult rats and mice. Our results show that adherens junctions adjacent to nGJs formed by Cx36 are composed of N-cadherin and nectin-1, and that these three proteins together with their interacting partners α-catenin, β-catenin, ZO-1 and AF6 are in close proximity or overlap to form what we refer to here as the adherens junction–nGJ (AJ–nGJ) complex.

EXPERIMENTAL PROCEDURES

Animals and antibodies

Animals used in this study included a total of twelve adult male Sprague–Dawley rats greater than two months of age and weighing 200–275 g, and fifteen adult male wild-type C57 BL/6-129SvEv mice greater than six weeks of age and weighing 25–30 g. An additional two C57 BL/6-129SvEv mice used included two transgenic Cx36 null animals, colonies of which were established at the University of Manitoba through generous provision of wild-type and Cx36 null breeding pairs (Deans et al., 2001) from Dr. David Paul (Harvard). Both rats and mice were used because data from early literature related to the present work were often derived from rat tissues, and more recent similarly related studies involved the use of mice. 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 stress and the numbers of animals used. Tissues from some animals were taken for use in other studies, thereby contributing to reduction in the total number of rats and mice used by laboratory personnel in various unrelated studies.

Immunofluorescence studies were conducted with the list of monoclonal and polyclonal primary antibodies given in Table 1, with western blotting and/or immunolabeling validation of antibody specificity for target proteins provided by the commercial supplier. Also included in Table 1 are the antibody catalog designations, the commercial source and the concentration or the dilution at which the antibodies were used during incubation with tissue sections. The polyclonal and monoclonal anti-Cx36 antibodies (ThermoFisher, Rockford, Il, USA; formerly Life Technologies Corporation, and originally Invitrogen/Zymed Laboratories) have been previously characterized for specificity of Cx36 detection in various regions of rodent brain, which has been confirmed using Cx36 null mice (Li et al., 2004a; Rash et al., 2007a,b; Curti et al., 2012). Secondary antibodies included Cy3-