Published in final edited form as:

Neuroscience. 2004 ; 126(3): 611–630.

CONNEXIN47, CONNEXIN29 AND CONNEXIN32 CO-EXPRESSION IN OLIGODENDROCYTES AND Cx47 ASSOCIATION WITH ZONULA OCCLUDENS-1 (ZO-1) IN MOUSE BRAIN

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Abstract

Gap junctions between glial cells in mammalian CNS are known to contain several connexins (Cx), including Cx26, Cx30 and Cx43 at astrocyte-to-astrocyte junctions, and Cx29 and Cx32 on the oligodendrocyte side of astrocyte-to-oligodendrocyte junctions. Recent reports indicating that oligodendrocytes also express Cx47 prompted the present studies of Cx47 localization and relationships to other glial connexins in mouse CNS. In view of the increasing number of connexins reported to interact directly with the scaffolding protein zonula occludens-1 (ZO-1), we investigated ZO-1 expression and Cx47/ZO-1 interaction capabilities in brain, spinal cord and Cx47-transfected HeLa cells. From counts of over 9000 oligodendrocytes labeled by immunofluorescence in various brain regions, virtually all of these cells were found to express Cx29, Cx32 and Cx47.

Oligodendrocyte somata displayed robust Cx47-immunopositive puncta that were co-localized with punctate labeling for Cx32 and Cx43. By freeze-fracture replica immunogold labeling, Cx47 was abundant on the oligodendrocyte-side of oligodendrocyte/astrocyte gap junctions. By immunofluorescence, labeling for Cx47 along myelinated fibers was sparse in most brain regions, whereas Cx29 and Cx32 were previously found to be concentrated along these fibers. By immunogold labeling, Cx47 was found in numerous small gap junctions linking myelin to astrocytes, but not within deeper layers of myelin. Brain subcellular fractionation revealed a lack of Cx47 enrichment in myelin fractions, which nevertheless contained an enrichment of Cx32 and Cx29.

Oligodendrocytes were immunopositive for ZO-1, and displayed almost total Cx47/ZO-1 co-localization. ZO-1 was found to co-immunoprecipitate with Cx47, and pull-down assays indicated binding of Cx47 to the second PDZ domain of ZO-1.

Our results indicate widespread expression of Cx47 by oligodendrocytes, but with a distribution pattern in relative levels inverse to the abundance of Cx29 in myelin and paucity of Cx29 in oligodendrocyte somata. Further, our findings suggest a scaffolding and/or regulatory role of ZO-1 at the oligodendrocyte side of astrocyte-to-oligodendrocyte gap junctions.

Keywords

connexin47; connexin32; connexin43; gap junctions; PDZ domains; glia

Gap junctions are localized at specialized cell-to-cell contacts of plasma membranes in which connexin (Cx) proteins form bidirectional intercellular channels that allow passage of ions and

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small molecules between cells (Goodenough et al., 1996; Kumar and Gilula, 1996; Willecke et al., 2002). In the CNS, astrocytes are extensively coupled by gap junctions (astrocyte-to-astrocyte gap junctions [A/A junctions]) that, based on ultrastructural observations, were reported to contain Cx26, Cx30 and Cx43 (Yamamoto et al., 1990a,b; Nagy et al., 1999, 2001). Also present are astrocyte-to-oligodendrocyte gap junctions (A/O junctions) that contain the former three Cxs on the astrocyte side, and Cx32 on the oligodendrocyte side (Li et al., 1997; Rash et al., 2000, 2001; Nagy and Rash, 2000; Nagy et al., 2003a). Recently, it was reported that oligodendrocytes also express Cx47 (Odermatt et al., 2003) as well as Cx29, with conflicting results indicating either that mutually exclusive populations of these cells contain Cx29 and Cx32 (Altevogt et al., 2002), or that many of these cells contain both of these Cxs (Nagy et al., 2003a,b). Moreover, while Cx47 was earlier reported to be expressed in neurons (Teubner et al., 2001) and more recently has been described as a myelin-related gene (Menichella et al., 2003), our initial observations indicated Cx47 targeting primarily to oligodendrocyte cell bodies, and only sparse association with myelin (Nagy et al., 2003b, 2004). Thus, the cellular and subcellular expression patterns of Cx47 and the localization and ultrastructural relationships of Cx47 to other glial Cxs in the CNS remain to be clarified.

In addition to containing multiple Cxs, it is now recognized that gap junctions in a variety of systems are associated with regulatory or structural proteins, and it has been reported that the c-terminus of a number of Cxs, including Cx43, Cx31.9, Cx45, Cx46 and Cx50, interact with the postsynaptic protein PSD95/*Drosophila* junction protein Disc-large/tight junction protein zonula occludens-1 (ZO-1) (Giepmans and Moolenaar, 1998; Toyofuku et al., 1998; Kausalya et al., 2001; Laing et al., 2001; Nielsen et al., 2002, 2003). Sequence similarities between the c-terminus of Cx47 and the PDZ-binding consensus motifs in these other Cxs suggest that it may also interact with ZO-1. Although there are no reports documenting the expression of ZO-1 in oligodendrocytes, the possibility of Cx47/ZO-1 interaction is raised by recent identification of much wider expression patterns of ZO-1 in the CNS than previously recognized, including association of ZO-1 with Cx36 at inter-neuronal gap junctions (Li et al., 2004; Rash et al., 2004).

To address some of the above issues, we investigated Cx47 expression in mouse CNS by light microscope immunohistochemical and biochemical approaches. In addition, the association of Cx47 with ZO-1 was examined in mouse brain and Cx47-transfected HeLa cells using immunofluorescence, co-immunoprecipitation (IP) and *in vitro* pull-down assay methods. We also used freeze-fracture replica immunogold labeling (FRIL) to investigate the distribution and co-localization of ZO-1, Cx47 and Cx32 on oligodendrocyte cell bodies and in the outer layers of myelin. Detailed ultrastructural analysis of Cx47 in relation to Cx29 and Cx32 in oligodendrocytes will be separately reported elsewhere.

EXPERIMENTAL PROCEDURES

Antibodies and animals

Details of the antibodies used in this study, including citations to previous descriptions of antibody specificity characteristics and the dilutions at which these antibodies were used, are listed in Table 1. All rabbit polyclonal and mouse monoclonal anti-Cx antibodies were obtained from Zymed Laboratories (South San Francisco, CA, USA), including a recently available antibody generated against a peptide corresponding to a sequence within the c-terminus of mouse Cx47, the characteristics of which has been previously described (Nagy et al., 2003b). Polyclonal goat anti-Cx32 and polyclonal goat anti-Cx43 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal and monoclonal anti-ZO-1 antibodies, and a polyclonal anti-ZO-3 antibody (36–4000) were obtained from Zymed Laboratories. Antibodies against oligodendrocyte markers included monoclonal anti-2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase; Sternberger Monoclonals, Baltimore, MD, USA), and

monoclonal anti-myelin-associated glycoprotein (MAG; Chemicon International, Temecula, CA, USA). Rabbit anti-glutathione-S-transferase (GST) antibody (Cat. No. 06-332) for detection of GST-PDZ domain fusion proteins was obtained from Upstate Cell Signaling Solutions (Lake Placid, NY, USA).

A total of 25 adult male CD1 mice and 12 male C57BL/6 mice were used in this study according to protocols approved by the Central Animal Care Committee, with minimization of stress to, and number of animals used. All experiments were conducted according to the "Principles of laboratory animal care" (NIH publication 86-23, revised 1985).

Western blotting and brain subcellular fractionation

Mice given an overdose of equithesin (3 ml/kg; Scadding, 1981) were decapitated, brains were removed for regional dissection on an ice tray, and tissue samples were rapidly frozen and stored at -80°C until future use. Brain tissues and cultured HeLa cells rinsed with phosphate-buffered saline (PBS; 50 mM sodium phosphate buffer, pH 7.4, 0.9% saline) were sonicated, homogenized in IP buffer (20 mM Tris-HCl, pH 8.0, 140 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EGTA, 1.5 mM MgCl_2 , 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 5 $\mu\text{g/ml}$ each of leupeptin, pepstatin A and aprotinin), and taken for Western blotting as previously described (Li et al., 1997, 2002; Nagy et al., 2003a,b). Briefly, following protein determination using a kit (Bio-Rad Laboratories, Hercules, CA, USA), 20–50 μg of protein per lane was separated electrophoretically by sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) using 12.5% gels in all studies, except in Fig. 2, where 10% gels were employed. Proteins were transblotted to polyvinylidene difluoride membranes (PVDF) membranes (Bio-Rad Laboratories) in standard Tris-glycine transfer buffer, pH 8.3, containing 0.5% SDS. Membranes were blocked for 2 h at room temperature in TBSTw (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.2% Tween-20) containing 5% non-fat milk powder, rinsed in TBSTw, and then incubated overnight at 4°C with either anti-Cx47 (1 $\mu\text{g/ml}$), anti-Cx32 (1 $\mu\text{g/ml}$), anti-Cx29 (0.5–1 $\mu\text{g/ml}$) or anti-CNPase diluted 1:2500 in TBSTw containing 1% non-fat milk powder. Membranes were then washed in TBSTw for 40 min, incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG or anti-mouse IgG diluted 1:3000–1:5000 (Sigma-Aldrich Canada, Oakville, Ontario, Canada) in TBSTw containing 1% non-fat milk powder, washed in TBST for 40 min and resolved by chemiluminescence (ECL; Amersham PB, Baie d'Urfe, Quebec, Canada).

Whole brains dissected from mice were taken for subcellular fractionation by discontinuous sucrose density gradient ultracentrifugation as previously described (Li et al., 1997; Lynn et al., 2001b; Nagy et al., 2003a). Material collected in the course of fractionation and at differing sucrose density interfaces included whole brain homogenate, soluble fraction, microsomal-plasma membrane fraction (P3), crude synaptosomal-mitochondrial fraction (P2), myelin fraction and synaptosomal fraction. Aliquots of each fraction were taken for Western blotting as described above, with equal inter-lane loading of protein.

Some variability in reaction of Cx47 antibody with non-specific proteins was encountered in the Western blots presented here, the reason for which remain uncertain, but may be due to slight differences in tissue preparation and/or experimental procedures that may occur despite efforts to maintain all parameters ideally the same.

Light microscope immunohistochemistry

Mice were deeply anesthetized with equithesin (3 ml/kg) and perfused transcardially with 3 ml of "prefixative" solution consisting of cold (4°C) 25 mM sodium phosphate buffer (PB), pH 7.4, 0.9% NaCl, 0.1% sodium nitrite and heparin (1 unit/ml). This was followed by perfusion with 20 or 40 ml of cold 0.16 M sodium PB, pH 7.6, containing either 1%, 2% or

4% freshly depolymerized para-formaldehyde and 0.2% picric acid, followed by perfusion with 10 ml of PB containing 10% sucrose. Brains were removed and stored at 4 °C for 48 h in cryoprotectant consisting of the final perfusate. Cryostat sections (10 µm thick) were collected on gelatinized glass slides, and processed for immunofluorescence staining with antibodies diluted, as indicated in Table 1, in 50 mM Tris–HCl, pH 7.4, containing 1.5% NaCl, 0.3% Triton X-100 (TBST) and either 4% normal goat serum or normal donkey serum. For single labeling, sections were incubated for 24 h at 4 °C with polyclonal rabbit anti-Cx47 (Ab36-4700), then washed for 1 h in TBST, and incubated for 1.5 h at room temperature with either Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) diluted 1:500, or Cy3-conjugated donkey anti-rabbit (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted 1:200.

For double immunofluorescence labeling, sections were incubated with polyclonal anti-Cx47 and simultaneously with either monoclonal anti-CNPase or monoclonal anti-ZO-1 for 24 h at 4 °C. For triple immunofluorescence labeling, sections were incubated simultaneously with antibodies against Cx47, Cx32 and CNPase, or Cx47, Cx43 and CNPase, or Cx32, ZO-1 and CNPase for 24 h at 4 °C. Sections were then washed in TBST for 1 h at room temperature and incubated for 1 h simultaneously with fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG diluted 1:200 (Jackson ImmunoResearch Laboratories) or Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR, USA) diluted 1:1000, in combination with Cy3-conjugated donkey anti-goat IgG and Cy5-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) diluted 1:200. Sections were then washed for 20 min in TBST, followed by two 20 min washes in 50 mM Tris–HCl buffer, pH 7.4, and coverslipped with antifade medium. Control procedures included omission of one of the primary antibodies with inclusion of each of the secondary antibodies to establish absence of inappropriate cross-reactions of primary with secondary antibodies.

For immunolabelling of HeLa cells transfected with Cx47 or empty vector, cells cultured on poly-L-lysine-treated glass coverslips were rinsed in PBS and fixed with ice-cold 1–2% formaldehyde for 5 min. Cultures were incubated overnight at 4 °C simultaneously with rabbit anti-Cx47 and mouse anti-ZO-1 in TBST containing normal donkey serum. Cultures were then washed in TBST for 1 h, then incubated for 1 h simultaneously with FITC-conjugated donkey anti-rabbit IgG diluted at 1:200 and Cy3-conjugated goat anti-mouse IgG diluted at 1:400, or alternatively, with FITC-conjugated horse anti-mouse IgG (Vector Laboratories Inc) diluted 1:200 and Cy3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories) diluted 1:400. Slides were then washed for 1 h in TBST and mounted with anti-fade medium.

Fluorescence was examined on a Zeiss Axioskop2 fluorescence microscope with image capture using Axiovision 3.0 software (Carl Zeiss Canada, Toronto, Ontario, Canada), and an Olympus Fluoview IX70 confocal microscope with image capture using Olympus Fluoview software. For confocal analysis, double- and triple-labeled sections were scanned twice or three times, respectively, using single laser excitation for each fluorochrome per scan. For generation of Z-stack images of Cx47-labeled oligodendrocytes, as series of 34 consecutive scans were taken through the z axis of single oligodendrocyte somata, and the final image was reconstructed with rotation in the x axis using Olympus Fluoview software. Images were assembled according to appropriate size and adjusted for contrast based on elimination of “empty” pixels using either Photoshop 6.0 (Adobe Systems, San Jose, CA, USA) or Corel Draw 8 (Corel Corp., Ottawa, Canada), and pseudocoloring was achieved using Northern Eclipse software (Empix Imaging, Mississauga, Ontario, Canada).

Counts of Cx-positive oligodendrocytes

Sections of mouse brain double-labeled by immunofluorescence for CNPase and either Cx29, Cx32 or Cx47 were used to assess quantitatively the percentage of oligodendrocytes that

displayed positive labeling for each of these Cxs. Counts of CNPase-positive oligodendrocytes that were Cx-positive or Cx-negative were undertaken in six different brain regions including cerebral cortex, hippocampus, thalamus, hypothalamus, brain stem and cerebellum. Sections were viewed at an objective magnification of $\times 20$ or $\times 40$ in a grid-like fashion that allowed examination of consecutively adjacent areas in a non-overlapping pattern. Only oligodendrocytes that displayed distinguishable and distinct punctate labeling on their somata were counted as Cx-positive. Data were collected from a total of eight mice, with $n = 3-5$ mice for counts of CNPase-positive cells that were positively or negatively labeled for each Cx (Cx29, Cx32 and Cx47) in each brain area examined. The mean percentage of CNPase-positive oligodendrocytes that were also labeled for oligodendrocyte Cxs in each animal was obtained by pooling counts from both sides of the brain for each brain region examined.

FRIL

Adult rats were anesthetized and fixed by perfusion with 1–4% formaldehyde in PB, cut into uniform 150 μm -thick sections using a Lancer 1000 Vibratome, frozen by contact with a liquid nitrogen cooled metal mirror, freeze-fractured and replicated, coated with Lexan plastic, thawed, photomapped, and cleaned with SDS detergent, as previously described (Pereda et al., 2003; Rash et al., 2001). Replicas with a thin layer of adhering molecules were immunogold labeled using polyclonal antibodies against Cx47, and monoclonal and polyclonal antibodies against ZO-1. Controls included examination of FRIL replicas in which primary antibodies to Cx47 were omitted and initial “blind” examination of some replicas. For replicas included in this study, “background” non-specific labeling was < 0.1 gold bead/ μm^2 and “signal” was > 300 / μm^2 of replicated gap junction, thereby minimizing the chance for “false positive” labeling. As illustration, we never detected a single gold bead for Cx47 labeling on gap junctions of astrocyte P-faces, even though astrocyte junctions are several hundred-fold more abundant than oligodendrocyte gap junctions (Rash et al., 1997, 2001), and therefore would be much more likely to yield false-positive labeling. Thus, even a single gold bead on an ultrastructurally defined gap junction constituted strong evidence for the presence of the target Cx in that gap junction. However, multiple gold beads for each target Cx were routinely found on larger gap junctions, and even the smallest gap junctions often contained two or more gold labels, and even greater numbers when labeled by 6 nm gold beads. Unfortunately, 6 nm gold beads are too small to be used reliably to detect gap junctions unless very high magnification examinations are employed. Labeled samples were rinsed and counter-labeled using goat anti-rabbit IgG and goat anti-mouse IgG, each coupled to a separate size of uniform-diameter gold beads (6, 12 and 18 nm; Jackson ImmunoResearch Laboratories). The 18 nm gold beads were applied for 18 h, whereas 6 nm gold beads were added for the last 2 h of secondary antibody labeling. Some samples were labeled with two sizes of gold beads for a single Cx. For example, 6 nm and 18 nm gold beads were used to label Cx47. This procedure allowed us to find small labeled gap junctions based on high visibility of 18 nm gold beads, but with the added advantage of ca. eight-fold higher labeling efficiency for 6 nm gold beads, thereby providing evidence for relative abundance of the target Cx in an individual gap junction, without necessity for tedious presentation of numerical data regarding “signal-to-noise ratio,” labeling efficiency for each size of gold bead, or methods for distinguishing false-positive and false-negative labeling (Rash and Yasumura, 1999). After rinsing, air-drying and carbon coating on the immunogold-labeled side of the replica, FRIL samples were examined in JEOL 2000 EX and JEOL 1200 transmission electron microscopes and photographed as stereoscopic pairs, with an 8° included angle.

Stereoscopic images are presented to allow discrimination between specific labeling and most forms of non-specific labeling, the latter of which occurs predominantly on the side opposite the immunogold labeled tissue remnants. Reverse stereoscopic images are also presented for one sample to allow easier detection of 6 nm gold beads. These small gold beads, which

otherwise may be difficult to discern beneath replicated intramembrane particles (IMPs), such as connexons, which are 10 nm in diameter and of greater electron opacity than the 6 nm gold beads.

Reverse transcription-polymerase chain reaction (PCR) and Cx47-pcDNA3 expression vector construction

Total RNA was isolated from adult mouse brain using the guanidine isothiocyanate method and reverse transcription using 500 ng oligo(dT)15 primer (Promega, Madison, WI, USA) was conducted as previously described (Lynn et al., 2001a; Li et al., 2002). Oligonucleotide primers, materials for amplifying mouse Cx47 coding sequence, and transfection reagents were purchased from Gibco BRL Life Technologies (Burlington, Ontario, Canada). Primers chosen for PCR were designed according to the mouse Cx47 sequence (Genbank accession number: NM_010290). The primer sequences were as follows: sense primer containing the kPn I restriction site, 5'-GGG GTA CCG ACC AAC ATG AGC TGG AGC TTC C -3'; antisense primer containing the *Bam*HI restriction site: 5'-GCA GGA TCC TCA GAT CCA CAC GGT GGC -3'. The PCR was carried out in 20 μ l of solution containing 2 μ l of 10 $\times$ PCR buffer, 0.8 μ l of 50 mM MgCl₂, 200 μ M dNTP, 100 ng sense and antisense primers, one unit of *Taq*DNA polymerase and 1 μ l of template cDNA. The PCR conditions were 94 °C for 3 min, then 35 cycles of amplification with 94 °C for 60 s, 62 °C for 60 s, and 72 °C for 90 s. This was followed by a final extension at 72 °C for 10 min for T-A cloning. PCR products were separated by electrophoresis in a 1% agarose gel, stained with ethidium bromide and purified using a gel purification kit (Qiagen Inc, Mississauga, Ontario, Canada). PCR products were subcloned into PCR 2.1 vector, digested with *Kpn*I and *Bam*HI, and ligated into pcDNA3 expression vector using T4 DNA ligase according to manufacturer's instruction. Recombinant plasmids were extracted, and at least two plasmids were sequenced using T7 universal primer and specific Cx47 primers for confirmation of sequence.

Cell culture and HeLa cell transfection

HeLa cells (American Type Culture Collection, Rockville, MD, USA) were grown in Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. For transient transfection, HeLa cells were transfected with pcDNA3 vector or Cx47-pcDNA3 plasmids using LipofectAMINE 2000 reagent as previously described (Li et al., 2002). For selection of stably transfected clones, cells 24 h after transfection were passaged at 1:40 dilution with fresh Dulbecco's Modified Eagle's medium containing 1.0 mg/ml of G418, and individual clones expressing Cx47 were isolated after 3 weeks in culture.

IP

Various regions of mouse brain (hippocampus, hypothalamus, cerebellum, midbrain, medulla), and Cx47-transfected HeLa cells were homogenized in IP buffer as previously described (Zhurinsky et al., 2000). Homogenates were sonicated and centrifuged at 20,000 $\times$ g for 10 min at 4 °C. Supernatants (2 mg) were pre-cleared for 1 h at 4 °C using 20 μ l of protein A-coated agarose beads (Santa Cruz BioTech), centrifuged at 20,000 $\times$ g for 10 min at 4 °C, and then incubated with 2 μ g of anti-ZO-1 antibody for 16 h at 4 °C. The mixture was then incubated for 1 h at 4 °C with 20 μ l of protein-A coated agarose beads, centrifuged at 20,000 $\times$ g for 10 min, and the pellet was washed five times with 1 ml of wash buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, and 0.5% NP-40). Samples were then mixed with an equal volume of SDS-PAGE loading buffer (125 mM Tris-HCl, pH 6.8; 20% glycerol; 0.3 mM Bromophenol Blue; 0.14 M SDS; 20% β -mercaptoethanol), then boiled for 3 min, and taken for immunoblotting with anti-Cx47 antibody. Control samples were precipitated with omission of anti-ZO-1 antibody.

GST-PDZ domain fusion proteins and *in vitro* pull-down assays

Three pGEX-3X plasmids each containing one of the three GST-linked PDZ domains of ZO-1 (Nielsen et al., 2002, 2003) were kindly provided to us by Dr. B. Giepmans (University of California, San Diego, CA, USA). Preparation of GST-PDZ fusion proteins from these plasmids expressed in *Escherichia coli* DH5 α and binding of fusion proteins to glutathione-agarose beads has been previously described (Li et al., 2004). These beads containing PDZ domain fusion proteins were incubated for 16 h at 4 °C with brain tissue or Cx47-transfected HeLa cells that had been homogenized in IP buffer. After extensive washing in PBS buffer containing 1% Triton X-100, proteins from the agarose beads were eluted with SDS-PAGE loading buffer, separated by SDS-PAGE, transferred to PVDF membrane and analyzed by Western blotting with anti-Cx47 or anti-GST antibody.

RESULTS

Cx47 regional expression and subcellular distribution

The regional expression of Cx47 in adult mouse brain was assessed by immunoblotting whole homogenates of various brain areas using 12.5% polyacrylamide gels (Fig. 1). In all brain regions examined, anti-Cx47 Ab36-4700 detected a protein band migrating at about 49 kDa. Additional faint bands detected at higher and lower molecular weights, particularly in thalamus and medulla, are of unknown identity. The 49 kDa band observed in brain corresponded to the migration profile of Cx47 detected by Ab36-4700 in HeLa cells stably transfected with Cx47. In comparison, homogenates from control HeLa cells transfected with empty vector showed absence of Cx47 detection, indicating specificity of the anti-Cx47 antibody. Very little variation in Cx47 levels was observed along the rostro-caudal axis of brain, including forebrain cerebral cortex to subcortical areas to hindbrain medullary regions, although hypothalamus contained slightly lower Cx47 levels than other structures. Immunoblotting showed an absence of Cx47 in sciatic nerve, even with high levels of protein loading and long immunoblot-to-film exposures (not shown).

The subcellular distribution of Cx47 in comparison with that of Cx32 and Cx29 in adult mouse brain is shown in Fig. 2. In immunoblots of subcellular fractions examined using 10% polyacrylamide gels, Cx47 migrated slightly slower at 51–53 kDa than seen in 12.5% gels, and appeared as a doublet of bands, possibility due to differential mobilities of Cx47 arising from post-translational modifications, similar to the altered migration patterns of various phosphorylated forms of Cx43 (Musil and Goodenough, 1991; Hossain et al., 1994). In subcellular fractions examined on 12.5% gels, the relative levels of Cx47 in the various fractions was similar to that shown in Fig. 2A, but only a single band was evident with migration profile corresponding to that seen in brain regions and in HeLa cells (not shown). The highest levels of Cx47 appeared in the microsomal P3 fraction, and in the synaptosomal fraction which is known to contain glial membrane contaminants as well as elevated levels of astrocytic Cxs (Lynn et al., 2001b). Curiously, and unexpected given the membrane localization of Cxs, moderate levels were also observed in the soluble fraction, suggesting a readily releasable, possibly intracellular pool of Cx47 that may have gone undetected by immunohistochemistry.

The lowest levels of Cx47 were observed in the crude synaptosomal–mitochondrial fraction, and in the myelin fraction where it was barely detectable. In contrast, Cx32 and Cx29 were most concentrated in the myelin fraction, with little evidence of their presence in the soluble fraction. In addition to recognition of the monomeric forms of Cx32 and Cx29, the anti-Cx32 and anti-Cx29 antibodies also detected bands migrating at 51 kDa and at 50 kDa, respectively, which likely correspond to the dimeric forms of these Cxs, as previously confirmed for Cx32 using WT and Cx32 knockout mice (Nagy et al., 2003b), and Cx29 transfected HeLa cells (Li et al., 2002). Designation of these bands as the dimeric forms of Cx32 and Cx29 is further

supported by their similar distribution profile in subcellular fractions, and similar levels of enrichment in the myelin fraction as the monomeric forms of these Cxs. Confirmation that membrane elements of myelin were successfully isolated and enriched during subcellular fractionation was provided by immunoblots showing that the myelin compared with all other fractions contained substantially greater levels of CNPase, which was previously reported to be a highly concentrated, insoluble component of myelin.

Immunofluorescence labeling of Cx47

In sections throughout adult mouse CNS, anti-Cx47 antibody produced robust immunolabelling of cell bodies having a distribution pattern and morphological appearance of oligodendrocytes (as confirmed ultrastructurally in results presented below). At low magnification, the minimal non-specific background labeling evident with this antibody readily revealed immunopositive cells having a moderate and even distribution in cerebral cortex, and a more dense distribution in gray matter of the thalamus and spinal cord (Fig. 3). Oligodendrocytes in white matter were also labeled for Cx47 (Fig. 3A, C), but generally more weakly than those in gray matter. Similar broadly distributed labeling of Cx47 in association with oligodendrocyte somata was observed in all CNS regions examined, including olfactory bulb, hippocampus, striatum, hypothalamus, midbrain and medulla (not shown). Optimal immunolabelling for Cx47 was achieved in brains of mice perfused with 2% formaldehyde/picric acid fixative with no post-fixation, and labeling was slightly or substantially suppressed using 4% formaldehyde without post-fixation or 4% formaldehyde with a 1.5 h post-fixation, respectively.

In sections double-labeled for the oligodendrocyte-myelin marker CNPase and for Cx47, it appeared that virtually all CNPase-immunopositive cells were also immunopositive for Cx47, as illustrated in the hippocampus (Fig. 4A, B) and the hypothalamus (Fig. 4C, D). While the low magnification fields shown in these areas contained an abundance of myelinated fibers as revealed by their immunolabelling for CNPase, these fibers were either unlabelled or displayed only faint, fine punctate labeling for Cx47. Other brain areas also displayed a sparse association of Cx47 with myelinated fibers in both gray and white matter (Fig. 3). Similar results were obtained in sections double-labeled for Cx47 and the oligodendrocyte-myelin marker MAG (not shown).

The cellular localization of Cx47 was further examined in relation to that of Cx32, which was previously shown to be concentrated in both oligodendrocyte somata and along myelinated fibers (Li et al., 1997; Nagy et al., 2003a,b). After double-labeling of sections derived from diverse areas of mouse brain, many oligodendrocyte somata found to be immunolabelled for Cx32 were also labeled for Cx47, as shown in cerebral cortex (Fig. 4E, F) and globus pallidus (Fig. 4G, H). Cx32 was also distributed along myelinated fibers in these (Fig. 4E, G) and other regions, whereas the same fibers displayed only scant labeling for Cx47 (Fig. 4F, H).

The percentage of CNPase-positive oligodendrocytes that were also positive for Cx29, Cx32 and Cx47 was quantitatively examined to determine whether subpopulations of oligodendrocytes differentially express the three Cxs. Greater than 9300 CNPase-positive oligodendrocytes in various brain regions were counted in sections simultaneously labeled and displaying the robust immunofluorescence for Cx47 or Cx32 shown in Figs. 3 and 4, and in those exhibiting the typically low but still identifiable labeling for Cx29 associated with oligodendrocyte somata (Nagy et al., 2003a). As shown in Table 2, 93% to 98% of CNPase-positive oligodendrocytes in the brain regions listed were found to be immunolabelled for Cx29, Cx32 and Cx47, suggesting that virtually all oligodendrocytes express all three Cxs.

Confocal immunofluorescence

Laser scanning confocal microscopy (Fig. 5) was used for detailed analyses of Cx47 immunolabelling associated with oligodendrocyte somata, and to determine Cx47 relationships with oligodendrocytic Cx32 and astrocytic Cx43. As can be gleaned from lower magnification images (Fig. 4), and readily evident by confocal immunofluorescence, labeling for Cx47 appeared exclusively as puncta decorating the periphery of oligodendrocyte somata and their initial processes. The density and spatial distribution of these immunopositive puncta on cells can be best appreciated in composite images of multiple Z-scans through single oligodendrocytes, with rotation of the image in the x axis. This is shown in the case of a Cx47-labeled oligodendrocyte, rotated 135° at 45° intervals (Fig. 5A), displaying greater than fifty Cx47-positive puncta associated with its somata and along short stretches of its initial processes.

In single confocal scans of oligodendrocytes labeled by triple immunofluorescence for Cx47 and Cx32, combined with labeling of CNPase for identification of oligodendrocytes, the vast majority of Cx47-positive and Cx32-positive puncta associated with these cells throughout mouse brain consistently displayed a high degree of co-localization, as shown in the cerebral cortex (Fig. 5B) and hypothalamus (Fig. 5C). As shown in cerebral cortex (Fig. 5D) and hippocampus (Fig. 5E), similar single confocal scans of triple immunofluorescence for Cx47 and astrocytic Cx43, combined with labeling for CNPase, indicated substantial co-association of Cx47-positive puncta with Cx43-positive puncta along the periphery and initial processes of oligodendrocytes, presumably reflecting localization of these Cxs to gap junctions between oligodendrocytes and astrocytes. However, due to the lack of Cx47 expression in astrocytes, together with the much greater abundance of Cx43 associated with gap junctions between astrocytes, only a small proportion of Cx43-positive puncta were co-associated with Cx47-positive puncta.

Co-localization of Cx47 with ZO-1 in brain and HeLa cells

Confocal double immunofluorescence for CNPase and ZO-1 revealed that CNPase-positive oligodendrocytes throughout mouse CNS were decorated with ZO-1-positive puncta, as shown by an example of an oligodendrocyte in the cerebral cortex (Fig. 6A). Low magnification double immunofluorescence for Cx47 and ZO-1, illustrated in hypothalamus (Fig. 6B), indicated that the vast majority if not all Cx47-positive oligodendrocytes were also immunopositive for ZO-1. Higher magnification confocal analysis demonstrated near total co-localization of Cx47-positive puncta with ZO-1 positive puncta on oligodendrocytes in cerebral cortex (Fig. 6C), as well as in other brain regions shown only by image overlays of Cx47/ZO-1 double labeling (Fig. 6D–F). Fine, punctate labeling for ZO-1 (Fig. 6B2, C2) was more widely and more densely distributed than labeling for Cx47 (Fig. 6B1, C1) in all brain regions, suggesting association of ZO-1 with other cell types and cellular structures. However, oligodendrocytes were often identifiable based simply on the greater size, labeling intensity and density of their ZO-1 associated immunopositive puncta, which was evident in sections double-labeled for CNPase and ZO-1 (Fig. 6A).

As expected from the above results demonstrating near total co-localization of Cx47 with both Cx32 and ZO-1, triple immunofluorescence labeling examined by confocal microscopy indicated substantial overlap of punctate labeling for Cx32 and ZO-1 along the periphery of CNPase-positive oligodendrocytes, shown in hippocampus (Fig. 6G) and the thalamus (Fig. 6H).

Double immunofluorescence labeling for Cx47 and ZO-1 in cultured HeLa cells is shown in Fig. 7. In cells stably transfected with Cx47, both Cx47 (Fig. 7A1) and ZO-1 (Fig. 7A2) immunoreactivity appeared as immunopositive puncta distributed around the periphery of

cells, particularly at points of cell-cell contact. Low magnification image overlay (Fig. 7A3) and confocal analysis (Fig. 7C) of cells in culture indicated near total co-localization of punctate labeling for Cx47 and ZO-1. HeLa cells transfected with empty vector were devoid of Cx47-immunopositive puncta (Fig. 7B1), and displayed only sparse, weak punctate labeling for ZO-1, suggesting that transfection with Cx47 results in an induction of ZO-1 expression. High background fluorescence was seen in transfected as well as control HeLa cells even after primary antibody omission (not shown), indicating non-specific attachment of secondary antibody, which was likely due to the relatively weak fixation conditions required for visualization of ZO-1.

Cx47 at oligodendrocyte gap junctions by FRIL

By FRIL, Cx47 was abundant in gap junctions on oligodendrocyte somata (Fig. 8A, B), and was present in gap junctions of all sizes, from those having < 30 connexons to those having > 500 connexons (inscribed boxes, Fig. 8A). In contrast to data resolvable by immunofluorescence microscopy, Cx47 was also observed in numerous small gap junctions on the outer layer of myelin (Fig. 8C) in adult rat spinal cord. This discrepancy between LM and FRIL data may be due to the ability of FRIL to detect the 0.03–0.1 μm diameter gap junctions consisting of 10–100 connexons on myelin surfaces. These minute junctions may be too small to have been detected by light microscope immunofluorescence. Also, as in the case of low Cx29 levels associated with oligodendrocyte cell bodies (Nagy et al., 2003a), sparsely distributed, fine Cx47-positive puncta seen along fibers in optimally fixed material were largely eliminated with increasing fixation strength, indicating narrow fixation thresholds and disproportionately greater suppression of Cx47 detection by immunofluorescence in very small gap junctions along myelinated fibers compared with large gap junctions on oligodendrocyte somata.

ZO-1 in tight junctions and gap junction by FRIL

By FRIL, immunogold beads representing ZO-1 labeling were abundant along margins linking capillary endothelial cells in samples labeled by both monoclonal and polyclonal anti-ZO-1 antibodies (Fig. 9). At high magnification, gold beads were localized to tight junction strands, as previously shown by Fujimoto (1995), presumably indicating that cytoplasmic ZO-1 molecules remain firmly bound to tight junction transmembrane proteins, which in turn strongly adhere to the platinum/carbon replica (Fujimoto, 1995). This positive control for ZO-1 labeling also allows comparison of the relative level of labeling under the conditions used in simultaneous labeling experiments on CNS tissues.

Polyclonal antibody labeling for ZO-1 was found at oligodendrocyte gap junctions, both on oligodendrocyte somata (Fig. 10A, B) and on the outer surface of myelin (Fig. 10C). In contrast to our observations by immunofluorescence, monoclonal anti-ZO-1 antibody failed to produce FRIL labeling of oligodendrocyte gap junctions. It should be noted that the sequence against which monoclonal anti-ZO-1 was raised (amino acids 334–634) contains the PDZ3 domain (amino acids 409–490) and the SH3 domain (amino acids 504–572) of ZO-1. Thus, it is possible that the monoclonal anti-ZO-1 epitope lies within one of these domains, which at gap junctions but not tight junctions, remain blocked by interacting proteins in procedures used for FRIL, but become exposed in those used for immunofluorescence.

Interaction of Cx47 with ZO-1

In order to determine whether Cx47 has direct or indirect molecular interaction capability with ZO-1, ZO-1 was immunoprecipitated from homogenates of various brain regions and from Cx47-transfected HeLa cells, and blots of immunoprecipitate protein were probed with anti-Cx47 antibody. As shown in Fig. 11A, Cx47 was detected after IP of ZO-1 from hypothalamus, cerebellum, midbrain and medulla, but not in a control sample of hypothalamus processed for

IP with anti-ZO-1 antibody omission. Levels of Cx47 detected in IP material reflect variability of IP with anti-ZO-1 antibody in the various brain regions, rather than comparative levels of Cx47 in these regions. Similar detection of Cx47 was obtained after IP of ZO-1 from Cx47-transfected HeLa cells (Fig. 11B, lane 3), whereas control IP with anti-ZO-1 omission showed an absence of Cx47 (Fig. 11B, lane 4). Blots of Cx47 immunoprecipitated from empty vector-transfected and Cx47-transfected HeLa cells with anti-Cx47 antibody are shown as negative (Fig. 11B, lane 1) and positive (Fig. 11B, lane 2) controls for Cx47 detection.

As further controls, Cx47-transfected HeLa cells probed with anti-ZO-1 showed lack of anti-ZO-1 cross-reaction (Fig. 11C, lane 1) with a band corresponding to Cx47 in these cells (Fig. 11C, lane 2). However, in HeLa cells and in thalamus, anti-ZO-1 antibody detected proteins of unknown identity migrating at about 31 kDa and 60 kDa, respectively, (Fig. 11C). No reaction with these proteins was seen with monoclonal anti-ZO-1 antibody (not shown). In addition to detection of ZO-1 in thalamus, a protein migrating at about 125–130 kDa was detected by polyclonal and monoclonal anti-ZO-1 in HeLa cells and thalamus, which may represent a cross-reaction with ZO-3, with which ZO-1 has sequence homology. In view of this potential cross-reaction, it should be noted that anti-ZO-3 antibody, which produced robust immunofluorescence labeling of ZO-3 in liver, showed no labeling comparable to that seen with polyclonal anti-ZO-1 in brain, except along blood vessels (not shown), indicating that immunofluorescence labeling obtained with anti-ZO-1 antibodies was not the result of ZO-3 detection.

Direct binding of Cx47 to ZO-1 was examined by *in vitro* pull-down assays using homogenates from brain and Cx47-transfected HeLa cells incubated with sepharose beads coupled to fusion proteins containing the GST–first PDZ domain of ZO-1 (PDZ1), GST–second PDZ domain of ZO-1 (PDZ2) or GST–third PDZ domain of ZO-1 (PDZ3). As shown in Fig. 12A (lanes 4–9), Cx47 was detected on immunoblots of brain and Cx47-transfected HeLa cell homogenate proteins eluted from beads coupled to the GST-PDZ2 domain of ZO-1, but was absent on blots of proteins eluted from beads coupled to the GST-PDZ1 and GST-PDZ3 domains of ZO-1, indicating direct and selective binding of Cx47 to the second PDZ domain of ZO-1. Immunoblots of Cx47 in homogenates of Cx47-transfected HeLa cells and brain were used as positive controls (Fig. 12A, lanes 2 and 3), and empty vector-transfected cells as negative controls (Fig. 12A, lane 1), for Cx47 detection. Immunoblot membranes shown in Fig. 12A (lanes 4–6 and 7–9) were stripped and reprobed with anti-GST antibody (Fig. 12B, corresponding lanes 1–3 and 4–6) to confirm the presence and equal loading of GST fusion proteins derived from the pull-down assays.

DISCUSSION

The present results confirm and extend previous findings of Cx47 expression in oligodendrocytes (Odermatt et al., 2003; Menichella et al., 2003; Nagy et al., 2003a,b), and not in neurons as originally reported (Teubner et al., 2001). In addition, we demonstrate subcellular sites of Cx47 targeting in oligodendrocytes, ZO-1 expression and gap junction localization in oligodendrocytes, Cx47/ZO-1 co-localization in oligodendrocytes and Cx47-transfected HeLa cells, and direct molecular interaction of Cx47 with the second PDZ domain of ZO-1.

With the identification of three different Cxs expressed in oligodendrocytes (Cx29, Cx32 and Cx47) and a different set of three Cxs expressed in astrocytes (Cx26, Cx30 and Cx43; see however Filippov et al., 2003; Condorelli et al., 2003), the apparently intricate gap junctional coupling relationships among these cells, and their Cx coupling partners in homotypic, heterotypic and potentially heteromeric combinations remains to be unraveled. In light of ultrastructural evidence indicating extensive gap junctions between astrocytes and between

astrocytes and oligodendrocytes, and the absence of intercellular gap junction formation between oligodendrocytes (Mugnaini, 1986; Rash et al., 1997, 2000), it is clear that the astrocytic Cxs participate in the formation of A/A junctions, and contribute these Cxs to the astrocyte side of A/O gap junctions. Less clear and requiring detailed ultrastructural investigation, however, is the extent to which oligodendrocyte cell populations express each of Cx29, Cx32 and Cx47; the oligodendrocyte cellular compartments in which these Cxs are concentrated; and the nature of the gap junctional channels in which they engage.

Cx29, Cx32 and Cx47 co-expression in oligodendrocytes

Our quantitative analyses revealed that 93–98% of CNPase-immunopositive cells displayed labeling for Cx29, Cx32, and Cx47, indicating that virtually all oligodendrocyte somata contain all three of these Cxs. Although a very small percentage of oligodendrocytes may lack one or the other of these Cxs, the less than total CNPase/Cx co-localization more likely reflects the limit of sensitivity of the immunohistochemical procedures used. This is in contrast to reports that oligodendrocytes differentially express Cx29 and Cx32 in a mutually exclusive fashion (Altevogt et al., 2002; Menichella et al., 2003), which would imply the existence of subclasses of oligodendrocyte populations differentiated by their Cx expression patterns. The relative abundance of the three oligodendrocyte Cxs at various cellular locations (somata vs. myelinating processes) is a separate issue (discussed below). The low levels of immunofluorescence labeling for Cx29 we observed along plasma membranes of oligodendrocyte somata (Nagy et al., 2003a,b), combined with low efficiency of labeling with anti-Cx29 antibodies or fixation-induced suppression of Cx29 detection, may produce results suggesting the absence of Cx29 on many oligodendrocyte cell bodies. Indeed, we have found that inclusion of a post-fixation step in CNS tissue preparation readily eliminates detection of Cx29 in oligodendrocyte somata, potentially accounting for the differing observations of Altevogt et al. (2002).

Cx29, Cx32 and Cx47 differential subcellular localization

Previous detailed comparisons of Cx29 and Cx32 cellular distributions in mouse brain (Nagy et al., 2003a) indicated that large Cx32-positive puncta were densely distributed on oligodendrocyte cell bodies and their initial processes, whereas minute Cx29-positive puncta were sparsely distributed, but partially co-localized with Cx32 on these cell bodies and absent on their initial processes. The present observations revealing similar distributions and near total co-localization of Cx32- and Cx47-positive puncta on oligodendrocytes and their initial processes are consistent with previous reports (Menichella et al., 2003; Nagy et al., 2003b), and our FRIL data suggest an abundance of these two Cxs in the same gap junctional hemiplaques on the oligodendrocyte side of A/O gap junctions (unpublished observations).

A number of other studies have demonstrated Cx29 and Cx32 along myelinated fibers, with a range of immunolabelling densities associated with varying proportions of fibers (Spray and Dermietzel, 1995; Li et al., 1997; Pastor et al., 1998; Scherer et al., 1995; Altevogt et al., 2002; Nagy et al., 2003a). In the most extreme case, Cx29 and Cx32 were reported to be localized to mutually exclusive subpopulations of fibers, which at face value appeared to be consistent with the reported expression of these Cxs by separate populations of oligodendrocytes (Altevogt et al., 2002). In contrast, we have found widespread localization of both Cx29 and Cx32 along myelinated fibers, which was confirmed for Cx32 by the absence of labeling along fibers in Cx32 knockout mice. In addition, many examples were found indicating localization of these Cxs on separate fibers, as well as co-localization on the same fibers (Li et al., 1997; Nagy et al., 2003a,b). While ultimately relevant to functions subserved by Cx29 and Cx32 in myelin, limits of light microscope resolution preclude assessment of the proportion and types of fibers containing either one or both of these Cxs, an issue which may be best approached by thin-section immunoelectron microscopy and FRIL.

The present immunofluorescence results demonstrate that myelinated fibers contain far less labeling for Cx47 than for Cx29 or Cx32, whereas FRIL revealed numerous small gap junctions containing Cx47 on the outer layer of myelin. Analyses of oligodendrocyte Cxs by subcellular fractionation of whole brain confirmed previous reports of an enrichment of Cx29 and Cx32 in the myelin fraction (Li et al., 1997; Nagy et al., 2003a) and supported our anatomical observations by showing relatively low levels of Cx47 in myelin, as compared with other fractions. Further evidence for the relatively low levels of Cx47 association with myelin compared with oligodendrocyte somata was indirectly provided by the progressively increasing density of myelin in the rostro-caudal direction of the neuraxis (Verity and Campagnoni, 1988), which was reflected by a similar rostro-caudal increasing gradient in Cx29 levels (Nagy et al., 2003a), but contrasted with the relatively uniform levels of Cx47 in all brain regions examined.

Several studies have reported that Cx29 and Cx32 are highly concentrated in Schmidt-Lanterman incisures at sites of uncompacted myelin in the peripheral nervous system (Scherer et al., 1995; Li et al., 2002; Altevogt et al., 2002), and it is possible that these Cxs may be at least partly associated with similar structures in the CNS (Nagy et al., 2003a). It is therefore noteworthy that the relatively low levels of Cx47 along fibers in the CNS was accompanied by an absence of Cx47 in peripheral nerves as observed by us (not shown) and others (Odermatt et al., 2003). This suggests a similar exclusion of Cx47 at incisures in both central and peripheral myelin, although this remains to be investigated ultrastructurally.

Astrocyte/oligodendrocyte Cx coupling

The expression of a distinct set of Cxs in astrocytes compared with those in oligodendrocytes suggests several possibilities for Cx coupling partners at A/O gap junctions on oligodendrocyte somata and at the outer surface of myelin, as well as at presumptive autologous gap junctions in non-compacted myelin. On the basis of relative Cx levels at these sites, Cx29 may contribute minimally to junctional coupling at oligodendrocyte somata (Nagy et al., 2003a), and Cx47 appears to contribute minimally to coupling within myelin. On the basis of Cx coupling permissiveness, the lack of Cx32 compatibility with Cx43 (White and Bruzzone, 1996) excludes participation of these Cxs in A/O gap junctions at oligodendrocyte somata or along myelin. Thus, Cx32 likely forms junctions with astrocytic Cx30 and/or Cx26, and Cx47 with one or more of the astrocytic Cxs at A/O gap junctions on oligodendrocyte somata, although permissiveness of Cx47 with Cx26, Cx30 and Cx43 has not been tested. Notably, levels of immunogold labeling for Cx47 and Cx43 were approximately equal in FRIL images of A/O junctions (J. E. Rash, unpublished observations). A prominent role of Cx32/Cx30 and Cx47/Cx43 combinations at these junctions was suggested by the large loss of astrocytic Cx30 and the persistence of both Cx43 and Cx47 at A/O gap junctions in Cx32 knockout mice (Nagy et al., 2003b). The lack of Cx29 permissiveness for formation of junctional channels with itself or with Cx32 (Altevogt et al., 2002) suggests Cx32 as the only contributor to presumptive autologous junction formation within myelin, and appears to leave Cx29 without a coupling partner in myelin. This raises the possibility that Cx29 forms exclusively hemichannels in myelin, as previously discussed in relation to the ultrastructural localization of Cx29 in peripheral nerve (Li et al., 2002), or that it forms channels with an as yet unidentified Cx. Alternatively, it has been suggested that Cx29 and Cx32 may form heteromeric channels, based on evidence that functional channels were formed between cells expressing Cx32 and those expressing a combination of Cx29 and Cx32 (Altevogt et al., 2002), raising the possibility that mixture of the two latter Cxs in individual connexons confers Cx29 with permissiveness for formation of functional channels with other Cxs. Whether this occurs *in vivo* remains to be determined.

The recently reported structural abnormalities in myelin sheaths of Cx32/Cx47 double knockout mice indicate essential functions of these Cxs in CNS myelin formation and/or maintenance (Odermatt et al., 2003; Menichella et al., 2003). The absence or relatively minor deficits in myelination observed in the CNS of either Cx32 or Cx47 knockout mice suggests functional compensation by other oligodendrocyte Cxs in these mice. However, it is possible that absence of some glial Cxs may give rise to physiological abnormalities that do not have overt structural correlates, as has been reported in Cx32 knockout mice (Bahr et al., 1999).

Cx47 association with ZO-1

Although originally defined as a tight junction-associated protein (Stevenson et al., 1986), and subsequently found at tight junctions in a wide variety of tissues (Gonzalez-Mariscal et al., 2003), ZO-1 is expressed by cells that lack tight junctions. It was shown to be a cytoplasmic component of adherens junctions (Itoh et al., 1991, 1993; Howarth et al., 1992), as well as gap junctions (Thomas et al., 2002). Studies of ZO-1 in the CNS have indicated restricted expression patterns, with localization to vascular, circumventricular and developing neuroepithelial cell types (Dermietzel and Krause, 1991; Howarth et al., 1992; Smith and Shine, 1992; Petrov et al., 1994; Aaku-Saraste et al., 1996; Saitou et al., 1997; Wolburg and Lippoldt, 2002; Vorbrodt and Dobrogowska, 2003), and a few types of neurons in the olfactory system and hippocampus (Miragall et al., 1994; Inagaki et al., 2003). However, we have found a much wider distribution of ZO-1 in the CNS, including its localization at inter-neuronal gap junctions composed of Cx36 (Li et al., 2004). The present demonstration of ZO-1 expression in oligodendrocytes further extends the list of CNS cell types that express this protein.

It is well established that oligodendrocytes form heterologous tight junctions with each other, and homologous or autologous tight junctions between the outer tongue of myelin and the underlying first layer of compact myelin (Dermietzel et al., 1978; Shinowara et al., 1980; Massa and Mugnaini, 1982; Mugnaini, 1986; Rash et al., 1997; Meier et al., 2004). The extent of ZO-1 association with these tight junctions remains uncertain. However, an association of one or more ZO proteins is likely based on reports that the tight junction forming protein claudin-11 is expressed by oligodendrocytes (Bronstein et al., 1996, 1997; Morita et al., 1999; Tiwari-Woodruff et al., 2001), contributes to tight junction formation in these cells as shown by the absence of tight junction strands in CNS myelin of claudin-11 knockout mice (Gow et al., 1999), and contains a consensus PDZ interaction motif that may directly bind ZO-1 (Morita et al., 1999; Gonzalez-Mariscal et al., 2003).

In addition to possible interaction of ZO-1 with claudin-11, we demonstrated that ZO-1 is localized at oligodendrocyte gap junction plaques and directly interacts with Cx47 via its second PDZ domain. Despite the growing number of Cxs reported to interact with ZO-1, including Cx43, Cx45, Cx31.9, Cx46, Cx50 (Giepmans and Moolenaar, 1998; Toyofuku et al., 1998; Kausalya et al., 2001; Laing et al., 2001; Nielsen et al., 2002, 2003), the function of this interaction and the significance of the differential binding of these Cxs to the second PDZ domain compared with the binding of Cx36 to the first PDZ domain of ZO-1 (Li et al., 2004) remains to be determined. Nevertheless, the importance of Cx47/ZO-1 interaction is suggested by the multifunctional, signaling and/or scaffolding role of ZO-1 in other cell types (Mitic and Anderson, 1998; Thomas et al., 2002; Gonzalez-Mariscal et al., 2003).

Acknowledgements

This work was supported by grants from the Canadian Institutes of Health Research to J.I.N., and the National Institutes of Health grants NS31027/NS39040 to JER. We thank B. McLean and N. Nolette for excellent technical assistance, and Dr. B. Giepmans for providing GST-PDZ expression vectors.

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Abbreviations

A/A junctions

astrocyte-to-astrocyte gap junctions

A/O junctions

astrocytes-to-oligodendrocytes gap junctions

CNPase

2',3'-cyclic nucleotide 3'-phosphodiesterase

Cx

connexin

FITC	fluorescein isothiocyanate
FRIL	freeze-fracture replica immunogold labeling
GST	glutathione-S-transferase
IMPs	intramembrane particles
IP	immunoprecipitation
MAG	myelin-associated glycoprotein
P2	crude synaptosomal-mitochondrial fraction
P3	microsomal-plasma membrane fraction
PB	phosphate buffer
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDZ domain	postsynaptic protein PSD95/ <i>Drosophila</i> junction protein Disc-large/tight junction protein ZO-1
PDZ1	the first PDZ domain of ZO-1
PDZ2	the second PDZ domain of ZO-1
PDZ3	the third PDZ domain of ZO-1
PVDF	polyvinylidene difluoride membranes
SDS	sodium dodecylsulfate
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis
TBST	Tris-HCl, pH 7.4, containing 1.5% NaCl, 0.3% Triton X-100
TBSTw	

10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.2% Tween-20

ZO-1

zonula occludens-1

Fig. 1.

Regional expression of Cx47 protein in adult mouse brain. Immunoblots show a migration profile of Cx47 in brain (lanes 1–7) comparable to that observed in Cx47-transfected HeLa cells (lane 9), and an absence of Cx47 in control empty vector-transfected HeLa cells (lane 8). Similar levels of Cx47 are seen in the various brain regions indicated (lanes 1–7). Additional higher and lower molecular proteins detected with moderate intensity in thalamus and medulla are of unknown identity.

Fig. 2.

Western blots showing the subcellular distribution of Cx47, Cx32 and Cx29 in adult mouse brain. Following subcellular fractionation, the same immunoblot loaded with equal levels of protein from whole brain homogenate (lane 1), soluble (lane 2), microsomal membrane (P3, lane 3), mitochondrial/synaptosomal (P2, lane 4), myelin (lane 5) and synaptosomal (lane 6) fractions were probed, stripped and reprobed for each of the Cxs (A, Cx47; B, Cx32; C, Cx29), and CNPase (D). Levels of Cx47 appear highest in the P3 fraction and lowest in the myelin fraction, whereas Cx32 and Cx29 are most concentrated in the myelin fraction. Enrichment of myelin in the myelin subfraction is indicated by elevated levels of the myelin-associated protein CNPase in this fraction (D, lane 5).

Fig. 3. Low magnification immunofluorescence micrographs showing the distribution of Cx47-immunopositive cells in various regions of adult mouse CNS. Labeling for Cx47 is seen associated with oligodendrocyte cell bodies in all layers of the cerebral cortex (A), in the corpus callosum (A, arrows), throughout the thalamus (B), and in all areas of gray matter (C) and white matter (C, asterisks) of the spinal cord. Oligodendrocytes in white matter appear more clustered and less intensely labeled than those in gray matter. Scale bars = 200 μ m (A–C, shown in C).

Fig. 4.

Double immunofluorescence micrographs showing relationships of cells labeled for CNPase, Cx32 and Cx47 in adult mouse brain. (A–D) The same fields in the hippocampus (A, B) and hypothalamus (C, D) showing correspondence of cells (arrows) labeled for the oligodendrocyte marker CNPase (A, C) and Cx47 (B, D). CNPase-positive fibers (A, C) appear either unlabelled or very faintly labeled for Cx47 (B, D). (E–H) The same fields in the cerebral cortex (E, F) and the globus pallidus showing correspondence of cells (arrows) labeled for Cx32 (E, G) and Cx47 (F, H). Some Cx32-positive fibers (E, arrowheads) appear only weakly labeled for Cx47 (F, arrowheads). Scale bars = 100 μ m (A, B, shown in B); C–H (shown in H), 50 μ m.

Fig. 5.

Laser scanning confocal immunofluorescence of Cx47-immunopositive puncta on CNPase-labeled oligodendrocyte somata, and their co-localization with Cx32 and Cx43. (A) Z-stack of 34 scans showing the same oligodendrocyte rotated through 135° at 45° intervals to reveal density of Cx47-positive puncta on somata and initial processes. (B, C) Confocal triple immunofluorescence showing individual oligodendrocytes in the cerebral cortex (B) and hypothalamus (C) labeled for Cx47 (B1, C1), Cx32 (B2, C2) and CNPase (B3, C3), with a high degree of Cx47/Cx32 co-localization as shown in overlay images (B4, C4). (D, E) Confocal triple immunofluorescence showing three oligodendrocytes in the cerebral cortex (D) and an oligodendrocyte in the hippocampus (E) labeled for Cx47 (D1, E1), Cx43 (D2, E2) and CNPase (D3, E3), with substantial Cx47/Cx43 co-association as shown in overlay images (D4, E4). Scale bars = 10 μ m.

Fig. 6. Immunofluorescence co-localization relationships of ZO-1 with CNPase, Cx47 and Cx32. (A) Confocal double immunofluorescence of a CNPase-positive oligodendrocyte somata (A1, arrow) displaying punctate labeling for ZO-1 (A2, arrow) as shown in overlay (A3). (B) Low magnification showing co-localization of Cx47 (B1) with ZO-1 (B2) on numerous oligodendrocytes (arrows) in the hypothalamus, as seen in image overlay (B3). (C) Higher magnification confocal micrographs showing Cx47-positive puncta (C1, arrows) and ZO-1-positive puncta (C2, arrows) on an oligodendrocyte in the cerebral cortex, with nearly total co-localization, as seen in image overlay (C3). (D–F) Confocal micrographs showing overlay images of Cx47/ZO-1 co-localization on oligodendrocyte in the thalamus (D), hypothalamus (E) and hippocampus (F). (G, H) Confocal triple immunofluorescence showing oligodendrocytes in hippocampus (G) and thalamus (H) labeled for Cx32 (G1, H1), ZO-1 (G2,

H2) and CNPase (G3, H3), with nearly total Cx32/ZO-1 co-localization as seen in image overlay (G4, H4). Scale bars = 10 μ m A; C–H; B, 20 μ m.

Fig. 7.

Immunofluorescence labeling of Cx47 and ZO-1 in cultured HeLa cells. (A) Double immunofluorescence of the same field showing punctate labeling of Cx47 (A1) and ZO-1 (A2) at points of cell-to-cell contact (arrows) in HeLa cells stably transfected with Cx47, and nearly total Cx47/ZO-1 co-localization, as seen by yellow in image overlay (A3, arrows). (B) Double immunofluorescence of the same field showing absence of labeling for Cx47 (B1) and faint, sparse labeling for ZO-1 at cell-cell contacts (B2) in control empty vector-transfected HeLa cells, and absence of punctate yellow labeling in image overlay (B3). (C) Confocal double immunofluorescence showing Cx47 (C1) and ZO-1 (C2) co-localized (C3) in individual immunopositive puncta at points of cell-cell contact between Cx47-transfected HeLa cells. Scale bars = 50 μ m (A, B); C, 10 μ m.

Fig. 8.

FRIL labeling of Cx47 in oligodendrocytes in adult rat spinal cord. (A) In a small area of oligodendrocyte soma, Cx47-immunoreactivity (12 nm gold) is seen in each of five gap junctions, which range from about 100 to more than 500 connexons. (B) At higher magnification, fifteen 12 nm gold beads label about 320 connexons (labeling efficiency approximately 1:20). (C) Cx47 (three and two 12 nm gold beads, white arrow) and Cx32 (one 18 nm gold, black arrow) were co-localized in two small gap junctions on the outer layer of myelin. In this image, one gap junction is labeled for Cx32. The gap junction at the lower left reveals both P- and E-face views, whereas the gap junction at the upper right is a P-face view, only.

Fig. 9.

Capillary in adult rat spinal cord after immunogold labeling for ZO-1. (A) At low magnification, the margins of five endothelial cells (E1–E5) reveal abundant 18 nm gold beads representing ZO-1 immunoreactivity. (B) In higher magnification stereoscopic images (B, left pair) and reverse stereoscopic images (B, right pair), both 6 nm gold beads (arrows) and 18 nm gold beads are seen in close proximity to tight junction strands. Scale bar = 0.1 µm.

Fig. 10.

FRIL localization of ZO-1 immunoreactivity at oligodendrocyte gap junctions (green overlays), but not at “reciprocal patches” (blue overlays). In images obtained from the same replica as Fig. 9, 6 nm and 18 nm gold beads are present at oligodendrocyte gap junctions, although the 6 nm gold beads are difficult to discern at these low magnifications. (A) Low magnification image of ZO-1-labeled gap junctions on oligodendrocyte soma. (B, C) Stereoscopic images (B is the inscribed area in A), revealing primarily 18 nm gold beads for ZO-1 beneath gap junction protoplasmic (P-) and extraplasmic (E-) faces. Gap junction P-faces consist of 10 nm IMPs, whereas E-face images of gap junctions have 6–7 nm pits separated by 10 nm. “Reciprocal patches,” which characterize oligodendrocytes, are an identifying

feature of oligodendrocyte plasma membranes, and are often intermixed with gap junctions (Rash et al., 1997), consist of mixed IMPs and pits on both P- and E-faces (Rash et al., 1998). At the lower left in C, the surface of myelin is continuous with cross-fractured myelin. Scale bar = 0.1 μ m, B, C.

Fig. 11.

Immunoblots showing co-IP of ZO-1 and Cx47 from various brain regions and from Cx47-transfected HeLa cells. (A) Homogenates of brain regions were taken for IP with anti-ZO-1 antibody and immunoblots of precipitates were probed with anti-Cx47 antibody. Cx47 is detected in precipitates of the brain areas indicated (lanes 1, 3, 4, 5), but not in control IP with anti-ZO-1 antibody omission (lane 2). (B) Lysates of control empty vector-transfected and Cx47-transfected HeLa cells show detection of Cx47 in the latter (lane 2), but not the former (lane 1). IP of ZO-1 from lysates of Cx47-transfected HeLa cells shows detection of Cx47 (lane 3), but not after IP with anti-ZO-1 omission (lane 4). (C) Control blots of Cx47-transfected HeLa cells and thalamus probed with polyclonal anti-ZO-1 (lanes 1 and 3) and anti-Cx47 (lane

2) showing absence of anti-ZO-1 cross-reaction with Cx47, but detection of unknown protein bands at 31 and 125–130 kDa in HeLa cells, and at 60 and 125–130 kDa in thalamus.

Fig. 12.

Pull-down assays showing Cx47 interaction with the second PDZ domain of ZO-1. (A) Immunoblots of lysates from empty vector-transfected HeLa cells (lane 1), Cx47-transfected HeLa cells (lane 2) and homogenate from brain tissue (lane 3) show negative and positive controls for Cx47 detection. Homogenates of brain and lysates of Cx47-transfected HeLa cells were incubated with GST fusion proteins containing either the PDZ1, PDZ2 or PDZ3 domain of ZO-1. Immunoblots of pull-down material from brain (lanes 4–6) and HeLa cells (lanes 7–9) show binding of Cx47 to the second PDZ domain of ZO-1 (lanes 5 and 8), but not to the first or third PDZ domains of ZO-1 (lanes 4, 6, 7, 9). (B) Membranes (lanes 4–9) were stripped

and re-probed with anti-GST antibody to confirm equal inter-lane loading of GST fusion protein.

Table 1

Antibodies used for Western blotting and immunohistochemistry

Antibody	Type*	Species	Epitope; designation ^a	Dilution	Reference; source
Cx29	Polyclonal	Rabbit	C-terminus; 34–4200	0.6 µg/ml	Li et al., 2002; Zymed
Cx32	Polyclonal	Rabbit	Loop; 71–0600	1.25 µg/ml	Li et al., 1997; Zymed
Cx32	Polyclonal	Rabbit	C-terminus; 34–5700	2.5 µg/ml	Zymed
Cx32	Polyclonal	Goat	C-terminus; sc-7258	2 µg/ml	Santa Cruz Biotech
Cx32	Monoclonal	Mouse	C-terminus; mAb3069	1:200	Chemicon
Cx43	Monoclonal	Mouse	C-terminus; 35–5000	2 µg/ml	Zymed
Cx43	Polyclonal	Goat	C-terminus; sc-6560	2 µg/ml	Santa Cruz Biotech
Cx43	Polyclonal	Rabbit	C-terminus; 71–0700	0.25 µg/ml	Li et al., 1998; Zymed
Cx43	Polyclonal	Rabbit	aa 346–363; 18A	1:1000	Yamamoto et al., 1990a,b
Cx47	Polyclonal	Rabbit	C-terminus; 36–4700	2 µg/ml	Zymed
ZO-1	Polyclonal	Rabbit	aa 463–1109; 33–9100	0.25 µg/ml	Zymed
ZO-1	Monoclonal	Mouse	aa 334–634; 61–7300	3 µg/ml	Zymed
CNPase	Monoclonal	Mouse	Whole protein	1:5000	Sternberger Monoclonals
MAG	Monoclonal	Mouse	Whole protein	1:500	Chemicon

^a aa, amino acids.

Percentage of CNPase-positive oligodendrocytes immunolabelled for Cx29, Cx32 or Cx47 in various brain regions of adult mice

Table 2

Brain area	Cx29-labelled oligodendrocytes	Cx32-labelled oligodendrocytes	Cx47-labelled oligodendrocytes
Cortex	96.6 ± 0.1 (735)	94.7 ± 1.1 (1389)	98.4 ± 0.2 (1637)
Hippocampus	95.4 ± 0.5 (206)	95.4 ± 0.5 (586)	97.1 ± 0.6 (518)
Thalamus	93.8 ± 0.6 (318)	94.9 ± 0.8 (704)	96.7 ± 0.5 (429)
Hypothalamus	93.0 ± 1.2 (259)	96.0 ± 0.8 (695)	94.1 ± 0.9 (420)
Brain stem	95.4 ± 0.7 (165)	93.9 ± 0.8 (291)	94.5 ± 0.7 (227)
Cerebellum	95.5 ± 0.5 (231)	95.7 ± 0.5 (258)	95.4 ± 0.6 (252)

Values represent means ± S.E.M. of the percentage of CNPase-positive oligodendrocyte cell bodies that displayed immunolabelling of Cx29, Cx32 or Cx47. Numbers in parentheses indicate the total number of CNPase-positive oligodendrocytes examined in each brain region of three to five C57BL/6 and CDI adult mice.