J Mol Neurosci
DOI 10.1007/s12031-007-9027-5

Gap Junctions Couple Astrocytes and Oligodendrocytes

Jennifer L. Orthmann-Murphy · Charles K. Abrams · Steven S. Scherer

Abstract In vertebrates, a family of related proteins called connexins form gap junctions (GJs), which are intercellular channels. In the central nervous system (CNS), GJs couple oligodendrocytes and astrocytes (O/A junctions) and adjacent astrocytes (A/A junctions), but not adjacent oligodendrocytes, forming a "glial syncytium." Oligodendrocytes and astrocytes each express different connexins. Mutations of these connexin genes demonstrate that the proper functioning of myelin and oligodendrocytes requires the expression of these connexins. The physiological function of O/A and A/A junctions, however, remains to be illuminated.

Keywords Connexins · Gap junctions · Glia · Myelin · K^+ buffering

Connexins Form GJs

In vertebrates, GJs are composed of connexins, a family of highly conserved integral membrane proteins that are usually named according to their predicted molecular mass (Willecke et al. 2002). As shown in Fig. 1, each connexin molecule is comprised of four transmembrane domains joined by two extracellular loops and one intracellular loop, with intracellular N- and C-terminal tails. Except for the intracellular loop and C-terminus, all of these regions are highly conserved (Yeager and Nicholson 1996). Six connexins oligomerize into a hemichannel (or connexon), and two hemichannels on apposing cell membranes form the intercellular channel, or GJ. Aggregates of tens to thousands of individual channels form a GJ plaque. Each channel permits the diffusion of ions and small molecules (typically less than 1,000 Da) between two apposed cells, enabling metabolic cooperation, spatial buffering, and electrical coupling (Bruzzone et al. 1996). The potential for diversity in the formation of GJs is immense, as over 20 mammalian connexins have been described (Willecke et al. 2002). As shown in Fig. 2, individual hemichannels can be composed of one (homomeric) or more (heteromeric) types of connexins. Similarly, GJ channels may contain hemichannels with the same (homotypic) or different (heterotypic) connexins (Bruzzone et al. 1996; Kumar and Gilula 1996). Some connexins may even preferentially form heteromeric rather than homomeric hemichannels (Plantard et al. 2003). Almost all connexins can form homomeric hemichannels and homotypic intercellular channels; Cx29 is one exception, as it may form hemichannels, but does not form functional homotypic channels (Altevogt et al. 2002; Ahn et al. 2008).

Like other intrinsic membrane proteins, connexins traffic within the secretory pathway to form GJs. Connexins are synthesized in the endoplasmic reticulum (ER), transported to the Golgi apparatus, and inserted into the cell membrane (Yeager et al. 1998). However, unlike most oligomeric transmembrane proteins (Rose and Doms 1988; Hurtley and Helenius 1989), multi-unit assembly of connexins may

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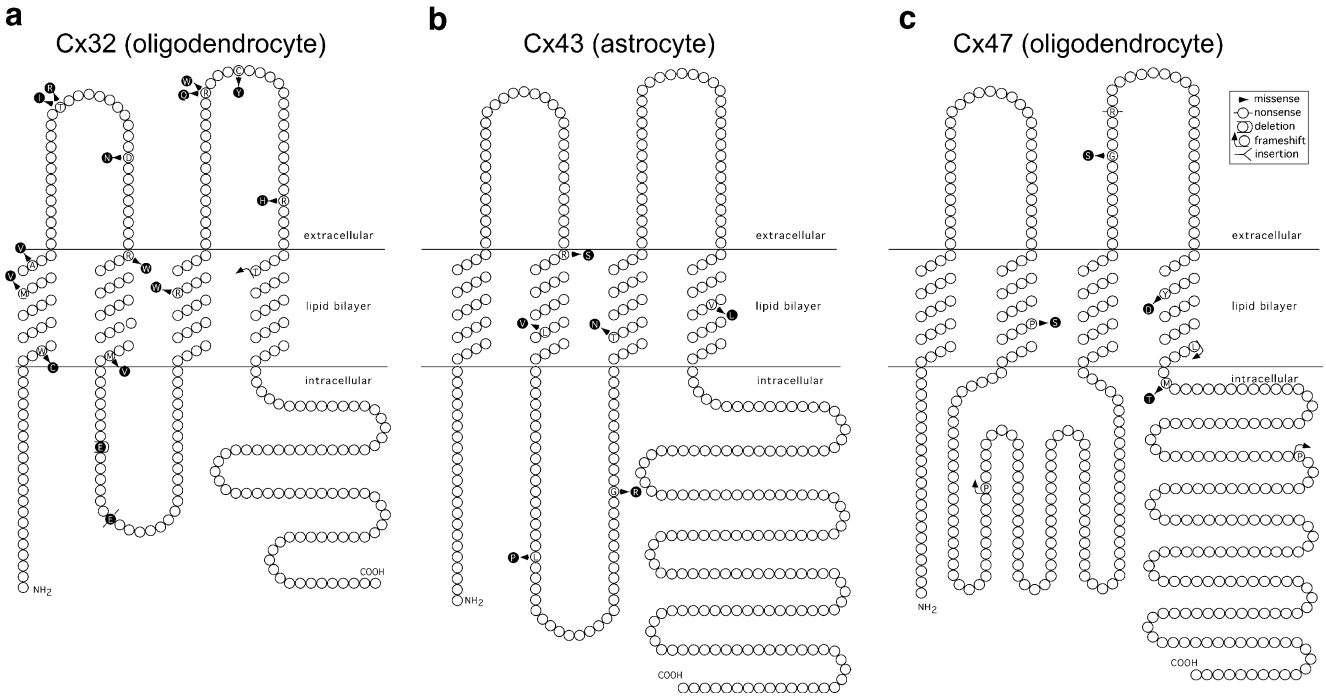

Figure 1 Cx32, Cx43, and Cx47 mutants associated with CNS disease. The structures of the connexin proteins are depicted according to Yeager and Nicholson (1996). Connexins have four transmembrane domains linked by two extracellular loops and one intracellular loop. Mutants associated with CNS disease are depicted in *black circles*. **a** The Cx32 mutants associated with a transient white matter disease in CMT1X are W24C, M34V, A39V, T55I, T55R, D66N, R75W, M93V, E102del, E109stop, R142W, R164W, R164Q, C168Y, R183H, and T191frameshift (Lee et al. 2002; Taylor et al. 2003). **b** The Cx43 ODDD mutants associated with well-documented CNS findings are R76S, L90V, L113P, G138R, T154N, and V216L (Opjordsmoen and Nyberg-Hansen 1980; Norton et al. 1995; Paznekas et al. 2003; Shapiro et al. 1997; Stanislaw et al. 1998; Wiest et al. 2006). **c** The recessive Cx47 mutants that cause PMLD are P87S, P128frameshift, G233S, R237stop, Y269D, L278frameshift, M283T, P327frameshift (Uhlenberg et al. 2004; Bugiani et al. 2006)

not be restricted to the ER. In cultured cells, endogenous Cx43 appears to oligomerize in a post-ER compartment, most likely the trans-Golgi network (Musil and Goodenough 1993), whereas Cx32 oligomerizes in the ER when expressed by transfection (Kumar et al. 1995; Das Sarma et al. 2002). Where other homomeric and heteromeric hemichannels oligomerize is largely unknown.

Any two compatible connexins can theoretically form 196 different channels (Brink et al. 1997), but functional and biochemical experiments have shown that not all connexin pairs are compatible (Bruzzone et al. 1996). Cx26 and Cx30, which are co-expressed in the cochlea (Kikuchi et al. 1995; Lautermann et al. 1998; Ahmad et al. 2003; Forge et al. 2003), comprise one of the best studied pairs of connexins that form heteromeric hemichannels. Both in vivo and in vitro, these two connexins colocalize to the same GJ plaques, co-immunoprecipitate, and form functional heteromeric hemichannels and heterotypic channels (Ahmad et al. 2003; Forge et al. 2003; Di et al. 2005; Sun et al. 2005; Yum et al. 2007). Yet even for this example, the subunit composition of heteromeric Cx26 and Cx30 channels and how this affects their physiological properties have not been resolved (Yum et al. 2007).

The suitability of connexins to form heterotypic channels is easier to study than their compatibility in forming heteromeric hemichannels (Cottrell and Burt 2005). In general,

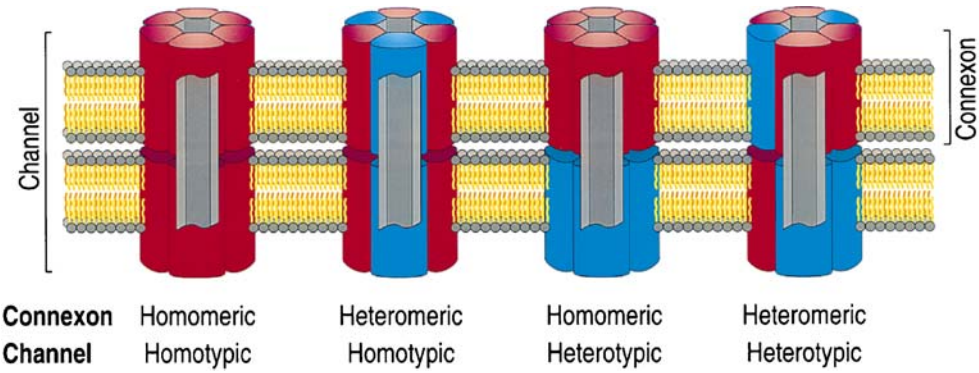

Figure 2 Diversity in GJ channel formation. This figure shows that two connexins (*red* and *blue*) can form GJ channels individually or in combination. Reprinted from (Kumar and Gilula 1996), with permission from Elsevier

only connexins that are closely related to each other can form functional heterotypic channels, although exceptions occur (Elfgang et al. 1995; White et al. 1995; Bruzzone et al. 1996). The compatibility of connexins is mostly determined by their two extracellular loops (White et al. 1995). Heterotypic channels formed by two different connexins can have functional properties that are distinct from their corresponding homotypic channels (Barrio et al. 1991; Bukauskas et al. 1995, 2002; Dahl et al. 1996; Valiunas et al. 2000; Harris 2007).

Gap Junctions and the Glial Syncytium

Figure 3 shows a schematic of the "glial syncytium" (Rash et al. 2001). There are abundant GJs between astrocytes themselves (A/A junctions), fewer between oligodendrocytes and astrocytes (O/A junctions), and few or none between oligodendrocytes themselves or between neurons and glia (Massa and Mugnaini 1982; Mugnaini 1986; Nagy et al. 2001; Rash et al. 2001; Kamasawa et al. 2005). Freeze-fracture electron microscopy has identified GJ plaques at A/A junctions between astrocyte processes and at O/A junctions on the oligodendrocyte cell body, processes, and outer (abaxonal) layer of the myelin sheath in both white and gray matter (Dermietzel et al. 1978; Massa and Mugnaini 1982; Waxman and Sims 1984; Yamamoto et al. 1990b; Rash et al. 1997, 2001). Examples are shown in Fig. 3. Although GJs form between adjacent oligodendrocytes in culture (Gonatas et al. 1982; Massa et al. 1984), if O/O junctions do exist in vivo, they are extremely rare (Mugnaini 1986; Rash et al. 2001).

Astrocytes are functionally coupled to other astrocytes and to oligodendrocytes by GJs. This has been shown both by electrophysiology (dual whole-cell patch clamp method) and by intercellular transfer of GJ permeant dyes (Kettenmann and Ransom 1988; Butt and Ransom 1989, 1993; Ransom and Kettenmann 1990; Robinson et al. 1993; von Blankenfeld et al. 1993; Venance et al. 1995; Pastor et al. 1998). Astrocytes in gray matter are generally strongly coupled, whereas

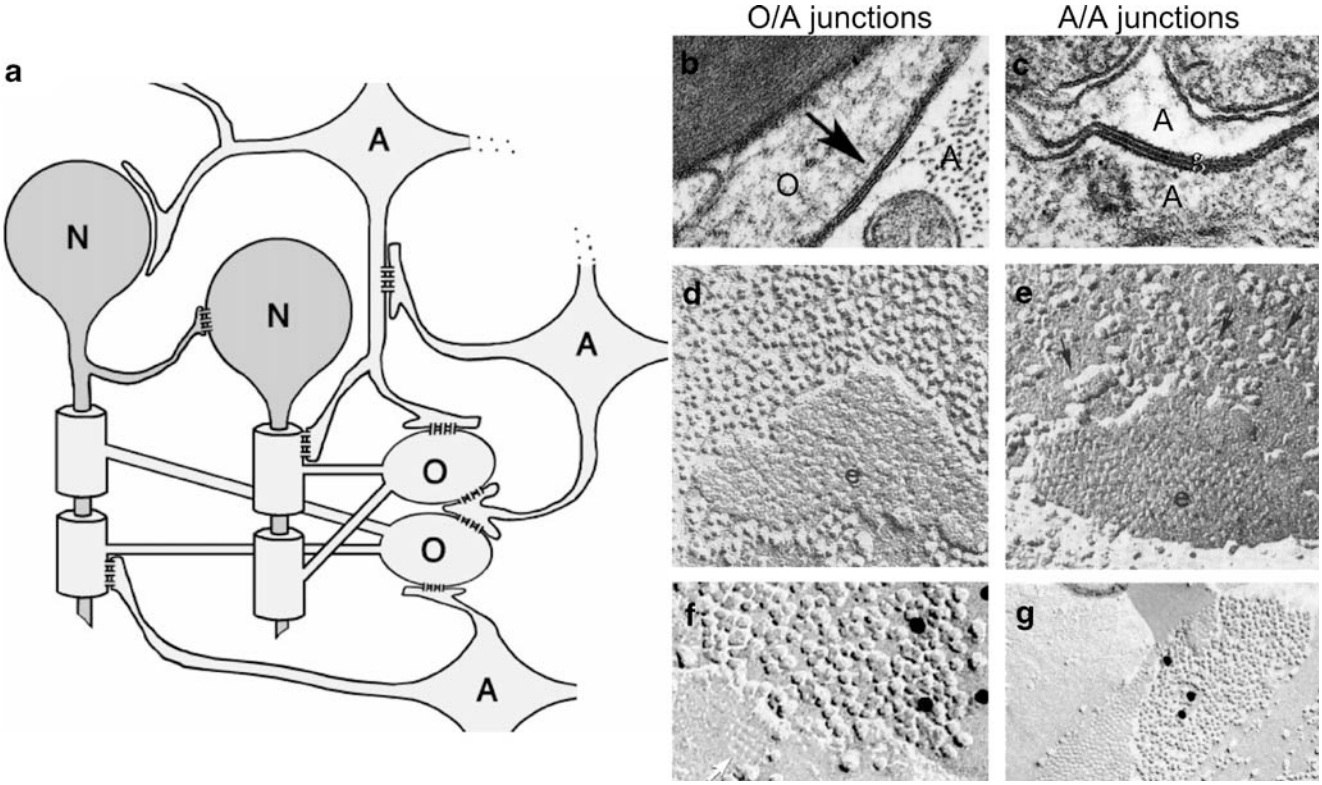

Figure 3 The "glial syncytium." **a** In this schematic depiction of the "glial syncytium," GJs join adjacent astrocytes, as well as adjacent astrocytes and oligodendrocytes, but not adjacent oligodendrocytes or adjacent neurons and glial cells. **b**–**g** Transmission (**b** and **c**) and freeze-fracture (**d**–**g**) electron microscopic images of GJs between adjacent glial cell membranes (*arrow* in **b**; *g* in **c**). **b** A GJ between an oligodendrocyte (O) and an astrocyte (A). **c** A GJ between two astrocytes. **d** An O/A GJ is composed of hundreds of individual channels in fractured apposed membranes. **e** An A/A junction; note that the pits of cleaved connexons are more densely packed in A/A (**e**) than in O/A junctions (**d**). **f** A FRIL image of an O/A junction immunogold-labeled for Cx47 (12-nm gold beads, *black dots*). **g** A FRIL image of an A/A junction immunogold-labeled for Cx43 (20-nm gold bead, *black dots*). *Square arrays* (orthogonal assemblies of aquaporin-4) identify astrocyte membranes in **e** (*black arrow*) and **f** (*white arrow*). Adapted from Rash et al. (2001; **a**), Mugnaini (1986; **b**, **d**, and **e**), Peters et al. (1991; **c**), Kamasawa et al. (2005; **f**), and Nagy et al. (2001; **g**), with permission from the Society for Neuroscience (**a**, copyright 2001), Elsevier (**b**, **d**, **e**, **f**), Oxford University Press (**c**), and Wiley–Liss (**g**)

astrocytes in white matter are varyingly coupled (Lee et al. 1994; Haas et al. 2006). Oligodendrocytes are coupled to astrocytes but probably not to adjacent oligodendrocytes. The rare finding of O/O coupling may be mediated by an interposed astrocytic process (Mugnaini 1986; Nagy et al. 2004). A/A and O/A coupling appears to increase over development (Butt and Ransom 1993), corresponding with the onset of connexin expression discussed below.

Robinson et al. (1993) reported that Lucifer yellow, a negatively charged dye, transferred preferentially from astrocytes to oligodendrocytes (and not in the reverse direction) in an intact retina. However, asymmetrical dye transfer violates the second law of thermodynamics (Finkelstein et al. 1994) and is unsubstantiated for compatible heterotypic channels tested in vitro (Elfgang et al. 1995; Weber et al. 2004). A likely explanation for this phenomenon is that the oligodendrocytes injected in this study were immature and not yet coupled to surrounding astrocytes (Hampson and Robinson 1995); alternatively, the dye injected into the oligodendrocytes was redistributed so quickly to the surrounding syncytium that transfer could not be detected.

Oligodendrocytes Express Cx29, Cx32, and Cx47

Immunofluorescence and freeze-fracture replica immunogold labeling (FRIL) assays have identified and localized the connexins expressed by oligodendrocytes in rodents. Oligodendrocytes express Cx32, Cx47, and Cx29 (Dermietzel et al. 1989; Micevych and Abelson 1991; Scherer et al. 1995; Li et al. 1997; Altevogt et al. 2002; Menichella et al. 2003; Nagy et al. 2003a, b; Odermatt et al. 2003; Altevogt and Paul 2004; Kleopa et al. 2004). Earlier reports that oligodendrocytes also express Cx45 (Dermietzel et al. 1997; Kunzelmann et al. 1997; Zahs et al. 2003) have been questioned (Kruger et al. 2000; Kleopa et al. 2004). Oligodendrocytes express Cx32, Cx47, and Cx29 messenger RNA (mRNA), and the timing of the mRNA and protein expression in the CNS parallels those of other myelin-related mRNAs and proteins (Scherer et al. 1995; Altevogt et al. 2002; Menichella et al. 2003).

Each oligodendrocyte connexin is uniquely localized, as shown in Figs. 4 and 5. Cx29 appears to be primarily expressed by small diameter myelinated fibers, which are found in both the gray matter and regions such as the corticospinal tract (Altevogt et al. 2002; Nagy et al. 2003a; Kleopa et al. 2004). Cx29 localizes to the inner/adaxonal membrane (apposing the axonal membrane) of small myelinated fibers, similar to its localization in Schwann cells (Altevogt et al. 2002). The adaxonal membrane does not form GJs with axons, however, and whether Cx29 forms functional hemichannels remains to be determined (Altevogt et al. 2002; Li et al. 2002; Kamasawa et al. 2005). Cx29 also localizes to the cell body of oligodendrocytes (Fig. 5), where it does not appear to form GJ plaques (Altevogt et al. 2002; Altevogt and Paul 2004; Kleopa et al. 2004; c.f. Nagy et al. 2003a). Cx32 is localized to oligodendrocyte cell bodies more prominently in gray than in white matter (Fig. 5), to the abaxonal membrane of primarily large myelinated fibers (Fig. 5), and between adjacent layers of myelin membrane at the paranode, where it most likely forms Cx32/Cx32 channels (Kleopa et al. 2004; Kamasawa et al. 2005). Whether these Cx32/Cx32 channels are restricted to the paranode remains unresolved; they may be localized in other regions of the myelin sheath, as shown previously in peripheral myelin (Scherer et al. 1995; Balice-Gordon et al. 1998;

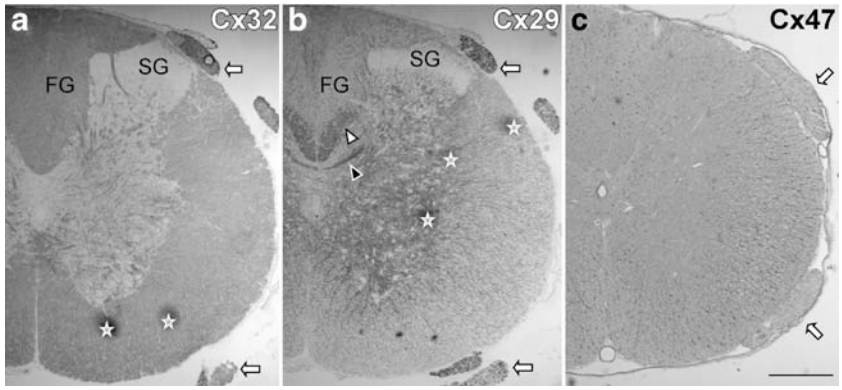

Figure 4 Differential expression of Cx32, Cx29, and Cx47. These are images of transverse sections of unfixed (**a** and **b**) or Zamboni-fixed (**c**) adult rat lumbar spinal cord using rabbit antisera against Cx32 (**a**), Cx29 (**b**), and Cx47 (**c**), visualized with a peroxidase-conjugated secondary antiserum. Cx32 immunoreactivity is mainly found in white matter tracts. Cx29 immunoreactivity is stronger in the *gray matter* and in fiber tracts that are enriched in small diameter fibers, such as the crossing posterior commissural fibers (*black arrowhead*) or corticospinal fibers (*white arrowhead*). Cx47 immunoreactivity is mainly present in oligodendrocyte perikarya, which are more numerous in the *white matter*. Cx32 and Cx29, but not Cx47, are strongly expressed in the spinal roots (*arrows*). *SG Substantia gelatinosa* and *FG Fasciculus gracilis*. *Asterisks* mark artifactual areas of staining. Scale bar, 200 μm. From Kleopa et al. (2004), with permission of Wiley–Liss

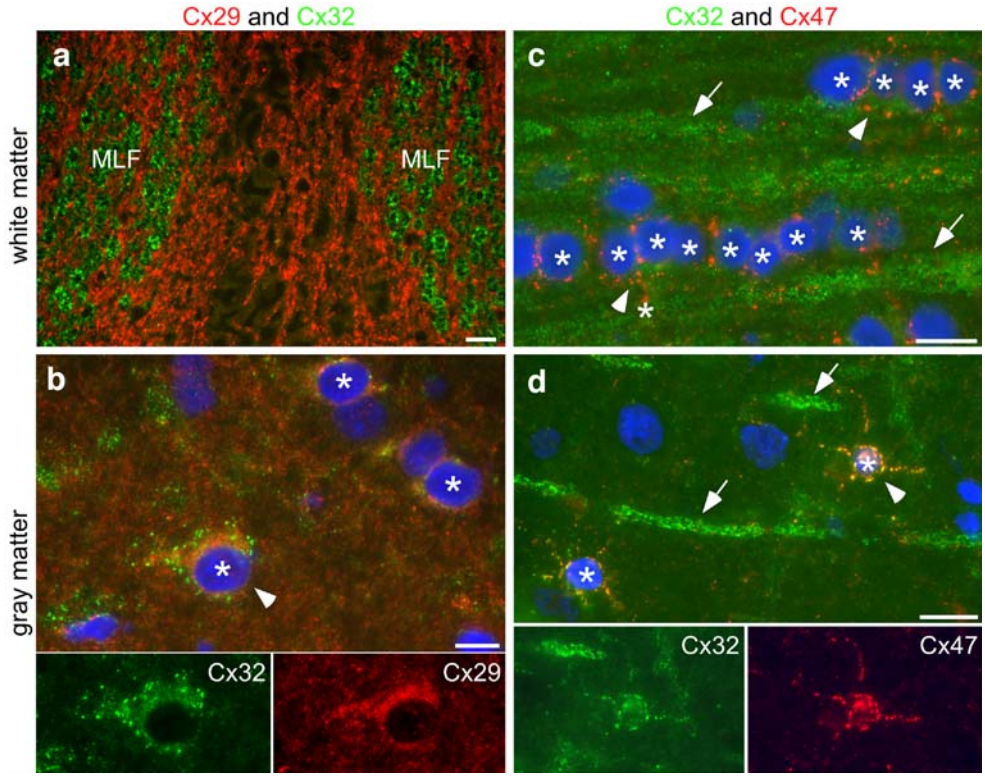

Figure 5 Differential distribution of Cx29, Cx32, and Cx47 in white and gray matter. These are images of unfixed rat CNS, immunostained with a rabbit antiserum to Cx29 (*red*; **a**–**b**), a mouse monoclonal antibody against Cx32 (*green*; **a**–**d**) or a rabbit antiserum to Cx47 (*red*; **c**–**d**), along with nuclear staining with 4′,6-diamidino-2-phenylindole (*blue* in **b**–**d**). In transverse sections of the pons (**a**), the large myelinated fibers of the medial longitudinal fasciculus (*MLF*) are Cx32-positive, whereas the surrounding smaller myelinated fibers are Cx29-positive. In the spinal cord gray matter (**b**), the same cell (*arrowhead*; also shown in the *bottom two panels*) has Cx29 and Cx32 immunoreactivity, but Cx29 is intracellular; it does not colocalize with Cx32 in GJ plaques. Oligodendrocyte perikarya (*asterisks*) in white matter (**c**; dorsal funiculus, longitudinal section) express Cx47 but not Cx32; those in gray matter (**d**; neocortex) express both Cx47 and Cx32. Note that there is substantial overlap between the Cx32 and Cx47 signals (*arrowhead*; also shown in the *bottom two panels*). Myelinated sheaths (*arrows*) express abundant Cx32 but little Cx47. Scale bars, 10 μm. These images are modified from Kleopa et al. (2004), with permission of Wiley–Liss

Meier et al. 2004). Cx47 localizes to cell bodies and initial processes of oligodendrocytes in both white and gray matter (Figs. 4 and 5; Nagy et al. 2003b; Altevogt and Paul 2004; Kleopa et al. 2004; Kamasawa et al. 2005). Cx32 and Cx47 have even been colocalized to the same plaque in the oligodendrocyte cell body and abaxonal membrane of myelinated fibers (Nagy et al. 2003b; Altevogt and Paul 2004; Kleopa et al. 2004; Kamasawa et al. 2005). Both connexins are detected on the oligodendrocyte aspect of O/A junctions in these locations (Li et al. 2004; Kamasawa et al. 2005; Rash et al. 2007a); an example for Cx47 is shown in Fig. 3f. Although FRIL shows that Cx47 localizes to many small GJ plaques in the abaxonal membrane of the myelin sheath (Kamasawa et al. 2005), Cx32 most likely constitutes the majority of channels here (Kleopa et al. 2004).

How Cx47, Cx32, and Cx29 localize to different aspects of the oligodendrocyte cell membrane is not understood. Proper localization may be mediated by interactions with particular scaffolding proteins expressed by oligodendrocytes. Cx47 has a PDZ-binding motif (on the intracellular C-terminus), which can interact with the second PDZ-binding domain of zonula occludens-1 protein (ZO-1), a scaffolding protein located at numerous junctional complexes (Giepmans and Moolenaar 1998; Li et al. 2004). In addition, Cx47 and ZO-1 colocalize at GJs in oligodendrocytes (Li et al. 2004). Because the PDZ-binding domain of Cx43 is required for binding ZO-1 but not for forming GJ plaques (Giepmans 2004), the Cx47/ZO-1 interaction may be required for localization at O/A junctions but not for its trafficking to the cell membrane. Cx32 is one of the few examples of a prenylated intrinsic membrane protein (Huang et al. 2005). Because prenylation-deficient Cx32 mutants properly localize in myelinating Schwann cells (Huang et al. 2005), the prenylation of Cx32 may not be required for its localization in oligodendrocytes. The paranodal localization of Cx32 in oligodendrocytes could be mediated by a complex that includes the tight junction protein claudin-11, as GJs are found in close proximity to tight junctions, which are prominent at paranodes (Sandri et al. 1982; Mugnaini 1986; Gow et al. 1999; Morita et al.

1999). Cx29 may be part of a complex that links juxtaparanodal proteins between axons and oligodendrocytes (Scherer et al. 2004).

Astrocytes Express Cx30 and Cx43

Astrocytes express Cx30 and Cx43 (Dermietzel et al. 1989; Yamamoto et al. 1990a; Micevych and Abelson 1991; Nagy et al. 1997, 1999, 2001, 2003b; Kunzelmann et al. 1999; Rash et al. 2001; Zahs et al. 2003; Altevogt and Paul 2004). Whereas Cx43 is expressed in early development in the nervous system and localizes throughout the white and gray matter in adult rodents (Dermietzel et al. 1989), Cx30 is expressed later (around postnatal week 4) and localizes primarily to gray matter (Dahl et al. 1996; Nagy et al. 1997, 1999, 2001; Kunzelmann et al. 1999). In the gray matter, Cx43 and Cx30 localize to the astrocyte aspect of O/A junctions, often colocalizing to the same GJ plaque; in the white matter, Cx43 predominates (Yamamoto et al. 1990a; Nagy et al. 1999, 2003b; Rash et al. 2001, 2007b; Zahs et al. 2003; Altevogt and Paul 2004). An example for Cx43 is shown in Fig. 3g.

There is uncertainty regarding whether astrocytes express Cx26. If they do, then Cx26 is restricted to the gray matter like Cx30 (Nagy et al. 2001, 2003b; Altevogt and Paul 2004). However, many studies that have examined Cx26 expression in astrocytes utilized an antibody that cross-reacts with Cx30 (Nagy et al. 1997, 2001; Altevogt and Paul 2004), which is highly homologous to Cx26. Further, the expression of *lacZ* in a Cx26 reporter mouse was not detected in astrocytes (Filippov et al. 2003) and the elimination of both Cx43 and Cx30 in astrocytes abolished A/A coupling in the mouse hippocampus (Wallraff et al. 2006). Thus, if astrocytes do express Cx26, then it is likely at very low levels.

Finally, glial cells may express pannexins, which show substantial homology with innexins, the proteins that form GJs in invertebrates (Phelan et al. 1998a, b; Panchin et al. 2000; White et al. 2004; Barbe et al. 2006). Pannexins are intrinsic membrane proteins with the same overall topology as connexins, but unlike connexins, pannexins are glycosylated (Penuela et al. 2007). Of the three mammalian pannexins, pannexin1 and pannexin2 are expressed in the CNS (Panchin 2005; Huang et al. 2007; Penuela et al. 2007). Cultured astrocytes, oligodendrocytes, and neurons express pannexin1 protein, but it is unresolved whether these cells express pannexin1 in vivo (Bruzzone et al. 2003; Ray et al. 2005; Vogt et al. 2005; Huang et al. 2007). Pannexin1 is unlikely to form functional channels (Huang et al. 2007; Penuela et al. 2007) but may form functional hemichannels (Locovei et al. 2006) or couple to other receptors (Pelegrin and Surprenant 2006).

O/A and A/A Gap Junctions

The above anatomical studies provide a framework for deducing the composition of the GJ channels at A/A and O/A junctions. By expressing each connexin in single cells (initially in oocytes), Cx30 and Cx43 were found to form homotypic channels (Swenson et al. 1989; Werner et al. 1989; Dahl et al. 1996). These studies suggested that A/A junctions might be comprised of Cx43/Cx43 and Cx30/Cx30 homotypic channels but did not evaluate the possible contributions of Cx43/Cx30 heterotypic channels or more complicated mixtures of heteromeric hemichannels.

O/A junctions must be heterotypic because the two cell types express different connexins; these channels may be comprised of Cx32/Cx30, Cx47/Cx43, Cx32/Cx43, or Cx47/Cx30. Electrophysiological studies established that Cx32/Cx30 channels (Dahl et al. 1996), but not Cx43/Cx32 channels (White et al. 1995), are functional. Although earlier studies in oocytes had suggested that Cx43/Cx32 channels were functional (Swenson et al. 1989; Werner et al. 1989), those studies did not suppress the expression of Cx38, which can form a functional channel with Cx43 (White et al. 1995). Furthermore, Cx30 and Cx32 are at least partly colocalized in the CNS, and Cx30 (but not Cx43) is mislocalized in *Gjb1/cx32*-null mice (Nagy et al. 2003b; Altevogt and Paul 2004). These authors also proposed that Cx47/Cx43 channels might contribute to O/A channels, but did not determine whether Cx43 is mislocalized in *Gj12/cx47*-null mice. Because Cx29 does not form GJ channels nor does it localize to GJ plaques in oligodendrocytes, it probably does not contribute to O/A junctions.

We tested which of these glial connexins can form heterotypic channels using dual whole-cell patch clamping (Orthmann-Murphy et al. 2007b). Pairs of mammalian cells, each of which expressed a single connexin (Cx30, Cx32, Cx43, or Cx47) were evaluated. As shown in Fig. 6, of the possible O/A heterotypic channel combinations, Cx32/Cx30 and Cx47/Cx43 formed functional channels; Cx32/Cx43 and Cx47/Cx30 did not. Similarly, of the possible A/A channel combinations, Cx30/Cx30 and Cx43/Cx43 formed functional channels, whereas Cx30/Cx43 did not. We also found that Cx32/Cx30 and Cx47/Cx43 channels exhibited distinct voltage-gating properties and permeabilities to GJ permeant dyes.

Based on the above findings, we proposed a cellular model of connexin channels in CNS glial cells. As shown in Fig. 7, A/A channels are mostly composed of Cx43/Cx43 channels, fewer Cx30/Cx30 channels (mostly in gray matter), and no Cx30/Cx43 channels. O/A channels are composed of Cx47/Cx43 and Cx32/Cx30 channels; both may be found in the same GJ plaque. Cx47/Cx43 channels are largely localized to oligodendrocyte somata, where they outnumber Cx32/Cx30 channels (Nagy et al. 2003b; Altevogt and Paul 2004;

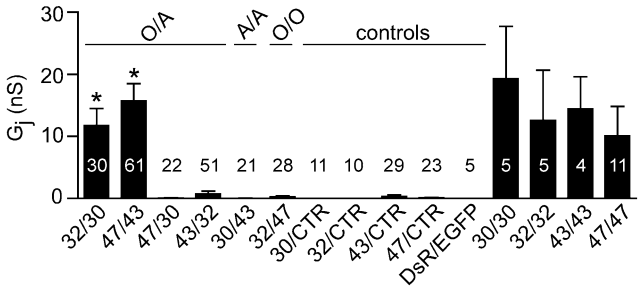

Figure 6 Cx32/Cx30 and Cx47/C43 form functional channels. Neuro2A cells were transiently transfected with pIRES2-EGFP or pIRES2-DsRed vectors containing human Cx30, Cx32, Cx43, Cx47, or no insert (vector alone control; CTR). After 24 h, EGFP- and DsRed-expressing cells were mixed in a 1:1 ratio; pairs were assessed by dual whole-cell patch clamping 6 to 48 h later. For each combination listed on the *x*-axis, the mean and standard error of the junctional conductance (G_j) and number of pairs tested are shown. Using Kruskal–Wallis test followed by Dunn's multiple comparison test, only Cx32/Cx30 and Cx47/Cx43 channels have G_j that is significantly greater ($p<0.001$; *asterisks*) than those of the corresponding control pairs (32/CTR and 30/CTR or 43/CTR and 47/CTR). From Orthmann-Murphy et al. (2007b), copyright 2007 by the Society for Neuroscience

Kleopa et al. 2004; Kamasawa et al. 2005). Cx32/Cx30 channels are mainly found on the outer layer of myelin sheaths and on oligodendrocyte cell bodies in gray more than in white matter (Kleopa et al. 2004; Kamasawa et al. 2005). Cx29 hemichannels localize to the internode along the adaxonal membrane of small myelinated fibers in both gray and white matter (Altevogt et al. 2002; Kleopa et al. 2004), and Cx32/Cx32 homotypic channels localize to paranodes (Kamasawa et al. 2005). If astrocyte (Cx43 and Cx30) or oligodendrocyte (Cx32 and Cx47) connexins form heteromeric hemichannels, then the composition of A/A and O/A channels would be more complex than depicted.

Connexins and Myelination

The strongest evidence that myelin function in the CNS is dependent upon the proper function of oligodendrocyte and astrocyte connexins comes from genetics. Mutations in the genes encoding Cx47 (*GJA12*) and Cx32 (*GJB1*) cause Pelizaeus–Merzbacher-like disease (PMLD) and the X-linked form of Charcot–Marie–Tooth disease (CMT1X), respectively (Fig. 1; note that updated connexin gene names are available at http://www.genenames.org/genefamily/gj.php). PMLD is a recessively inherited, devastating dysmyelinating disease (Uhlenberg et al. 2004; Bugiani et al. 2006), named because it clinically resembles PMD, which is caused by mutations in *Proteolipid Protein 1* (*PLP1*), the gene encoding the major intrinsic membrane protein of CNS myelin (Garbern et al. 1999). The PMLD patients described to date have similar phenotypes—nystagmus by 7 weeks, impaired motor development by 15 months, and evidence of hypomyelination on MRI (Uhlenberg et al. 2004; Bugiani et al. 2006). We recently showed that three of the missense mutations (P87S, Y269D, M283T) result in loss-of-function protein when expressed in a mammalian cell line (Orthmann-Murphy et al. 2007a). Unlike wild-type Cx47, these mutants were at least partially retained in the ER and failed to form functional homotypic channels. It is plausible that both recessive, partial loss-of-function mutations and dominant mutations in *GJA12/Cx47* will result in milder phenotypes than PMLD, but these mutations have not yet been described.

The above data suggest that loss-of-function Cx47 mutants most likely cause the PMLD phenotype by interfering with the normal function of Cx47/Cx43 channels. Indeed, all three missense mutants fail to form functional Cx47/Cx43 channels (Orthmann-Murphy et al. 2007b). Because mice lacking *Gja12/cx47* exhibit mild pathological changes in myelinated axons (Menichella et al. 2003; Odermatt et al. 2003), one would not have predicted that recessive mutations in humans would result in a devastating disease. The localization of Cx47 in humans has not been examined, so it is possible that the contribution of Cx47/Cx43 channels is different between rodents and primates, although oligodendrocytes in the rhesus monkey do express Cx47 (Orthmann-Murphy et al. 2007a). Alternatively, Cx32/Cx30 channels may be able to compensate for Cx47/Cx43 channels in rodents to a greater degree than in humans.

CMT1X is the second most common inherited demyelinating neuropathy (Scherer and Kleopa 2005). It is caused by more than 300 different mutations in *GJB1/Cx32* (http://www.molgen.ua.ac.be/CMTMutations/DataSource/MutByGene.

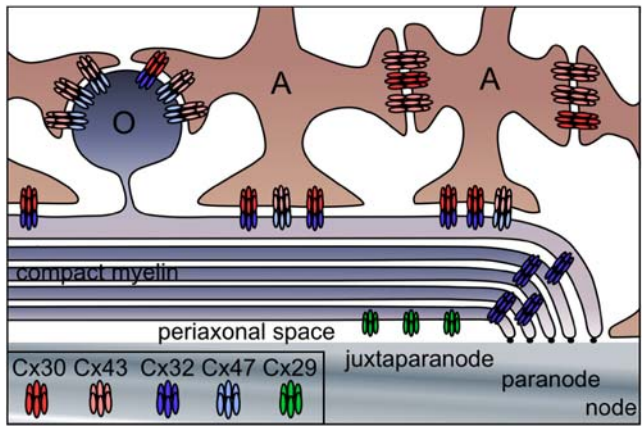

Figure 7 The gap junction network of astrocytes and oligodendrocytes. This drawing depicts a model for the GJ channels that connect astrocytes (A) to oligodendrocytes (O) and other astrocytes. A/A junctions contain Cx30/Cx30 and Cx43/Cx43 channels; O/A junctions contain Cx32/Cx30 and Cx47/Cx43 channels. Homotypic Cx32/Cx32 channels connect layers of myelin sheath at the paranode and incisures (not shown). Cx29 hemichannels are localized to the adaxonal membrane of oligodendrocytes, apposing the axon. From Orthmann-Murphy et al. (2007b), copyright 2007 by the Society for Neuroscience

cfm). Many mutations are associated with subclinical CNS findings, including central conduction slowing (Nicholson and Corbett 1996), but a few (Fig. 1) are associated with an acute and dramatic CNS phenotype, including weakness, paresis, and spasticity following an episode of acute stress (Lee et al. 2002; Taylor et al. 2003). We and others have shown that many mutant proteins appear to primarily localize intracellularly, to either the ER or Golgi, and do not form functional GJs (Abrams et al. 2000; Kleopa et al. 2002; Yum et al. 2002). Loss of Cx32 results in a demyelinating peripheral neuropathy in rodents, indicating that loss-of-function of Cx32 in Schwann cells is sufficient to cause this disease (Scherer et al. 1998). However, neither *Gjb1/cx32*-null mice nor *GJB1/Cx32*-null humans exhibit a CNS phenotype (Hahn et al. 2000), suggesting that another mechanism is responsible for the acute and/or subclinical findings described in CMT1X patients. Although Cx32 mutants may exert dominant negative effects, as shown in a transgenic mouse expressing both the R142W mutant and wild-type Cx32 in Schwann cells (Jeng et al. 2006), only one copy of *Gjb1/cx32* is present in males, and one copy is subject to X-inactivation in females (Scherer et al. 1998). Therefore, we have previously proposed that Cx32 CNS mutants transdominantly interact with another connexin expressed by oligodendrocytes (Kleopa et al. 2002). In a cell culture, these mutants did not interact with Cx45, but it remains to be shown whether CNS mutants interact with Cx47, and potentially disrupt O/A channels. In myelinating Schwann cells, the R142W mutant did not appear to alter the localization of Cx29 (Jeng et al. 2006).

Whereas loss of Cx32 or Cx47 alone does not cause an overt CNS phenotype in mice, loss of both results in a severe dysmyelinating phenotype (Menichella et al. 2003; Odermatt et al. 2003). These mice develop tremors and ataxia and typically die by the sixth postnatal week. There are various pathological alterations of myelinated axons, including vacuoles, especially between the axons and their myelin sheaths (Fig. 8). Recently, Menichella et al. (2006) provided the first direct evidence implicating neuronal activity in the development of myelin vacuoles in these double knockout mice. They found that increasing retinal ganglion cell activity (with intraocular cholera toxin) exacerbated vacuole formation in optic nerves, whereas inhibiting activity (with intraocular tetrodotoxin; Fig. 9) reduced vacuole formation (Menichella et al. 2006). To account for these findings, they proposed that O/A junctions are involved with the redistribution of K^+ that is elaborated during neural activity (see below).

Mutations in *GJA1/Cx43* cause oculodentodigital dysplasia (ODDD), a rare, dominantly inherited, syndromic disease associated with abnormalities in the face, eye, teeth, and digits (Paznekas et al. 2003). Some ODDD mutations (shown in Fig. 1) are also associated with a variety of CNS abnormalities, including spasticity and white matter lesions

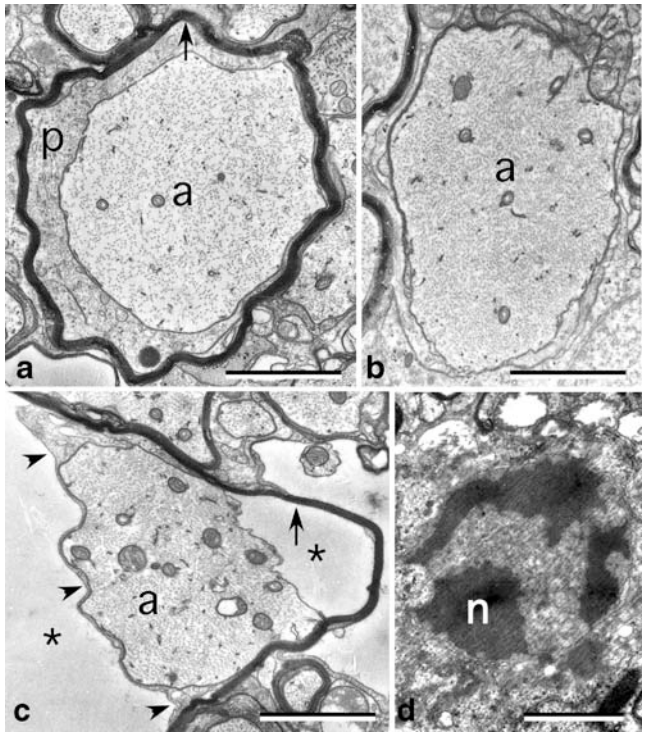

Figure 8 Pathological findings in *Cx32/Cx47* double null mice. These are electron micrographs of the ventral funiculus from a postnatal day 30 *Cx32/Cx47* double null mouse. **a** An axon (*a*) is surrounded by a periaxonal collar of cytoplasm (*p*) and a thin myelin sheath (*arrow*). **b** A demyelinated axon (*a*) has neurofilaments that are more tightly packed than in **a**. **c** An axon (*a*) is still partially surrounded by the adaxonal process of an oligodendrocyte (*arrowheads*) but is separated from its myelin sheath (*arrow*) by extracellular space (*asterisks*). **d** An apoptotic oligodendrocyte nucleus is shown. Scale bars: 1 μm. From Menichella et al. (2003), copyright 2003 by the Society for Neuroscience

(Loddenkemper et al. 2002; Paznekas et al. 2003). Although many of these CNS mutants can form GJ plaques when expressed in cultured cells, most do not form functional channels. Some mutants, furthermore, have a dominant negative effect on the function of wild-type Cx43 (S. S. Scherer, unpublished data; Seki et al. 2004; Roscoe et al. 2005; Shibayama et al. 2005; Gong et al. 2007). A dominant *Gja1/cx43* mutation has been discovered in mice (Flenniken et al. 2005), but it remains to be shown whether this missense mutation (G60S) results in CNS abnormalities. Because mice that express only one copy of *Gja1/cx43* (Reaume et al. 1995) exhibit none of the craniofacial deficits of the ODDD mouse, ODDD mutants probably cause more than a simple loss-of-function.

The K^+ Buffering Hypothesis

In spite of the genetic evidence that underscores the importance of Cx32, Cx43, and Cx47 in the CNS, the functional role of glial GJ channels is unresolved. GJs

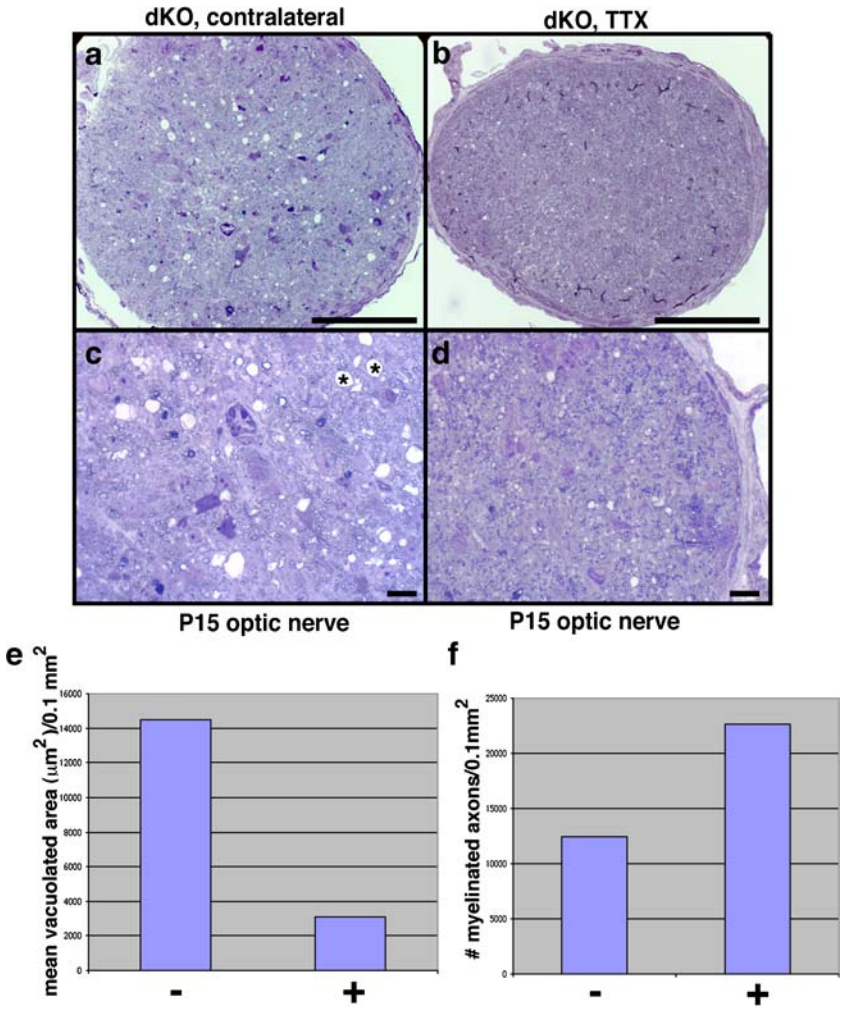

Figure 9 Suppressing axonal activity reduces vacuolation in optic nerves from *Cx32/Cx47* double null mice. Intraocular injections of tetrodotoxin (TTX) were performed at P11 and P13; optic nerves were harvested at P15. (**a**–**d**) Semi-thin sections from the optic nerve associated with an injected eye reveal a dramatic reduction in vacuolation compared to the contralateral control nerve. Scale bars: *top row* (**a**, **b**), 100 μm; *bottom row* (**c**, **d**), 10 μm. (**e**–**f**) Quantification of small (<20 μm^2) and large (>20 μm^2) vacuoles, as well as the total vacuolated area (μm^2/0.1 mm^2) and the number of myelinated axons (per 0.1 mm^2) in TTX injected (+) and uninjected/contralateral (−) optic nerves. TTX injections lead to fewer vacuoles and a reduction in total vacuolated area. Error bars indicate SEM. From Menichella et al. (2006), copyright 2006 by the Society for Neuroscience

transmit action potentials in cardiac muscle and certain neurons (Connors and Long 2004; Rohr 2004) but not in astrocytes or oligodendrocytes, which have only passive membrane currents (Chvatal et al. 1995). Since the landmark paper of Orkand and Nicholls (1966), the leading hypothesis has been that glial GJs spatially buffer K^+. Astrocytes slowly depolarize after stimulation of amphibian optic nerves because of rising extracellular K^+. Because they are permeable to K^+ and coupled by GJs, astrocytes could theoretically redistribute extracellular K^+ without significantly altering the intracellular [K^+] in any one cell. In isolated adult rodent optic nerves, K^+-sensitive electrodes demonstrate that the local $[K^+]_o$ rise is limited to ~10–12 mM after electrical stimulation (Connors et al. 1982; Ransom et al. 1985). Yet, even after more than 40 years of study (Orkand 1986; Walz 2000; Kofuji and Newman 2004), it is still unclear whether spatial buffering of K^+ has a critical role in the CNS.

In support of such a role, elevation of extracellular K^+ in primary astrocyte cultures or optic nerve stimulation in the frog appears to enhance glial GJ coupling (Enkvist and McCarthy 1994; Marrero and Orkand 1996). In addition, Holthoff and Witte (2000) showed that intracellular acidification (which tends to close GJ channels) or treatment with carbenoxolone (a nonspecific blocker of channels including GJs) blocked local shrinkage and distant expansion of the extracellular space after electrical stimulation in slices of rat neocortex (Holthoff and Witte 2000). However, a recent study by Wallraff and colleagues demonstrated that eliminating both Cx30 and Cx43 in astrocytes modestly diminished, but did not abolish, K^+ buffering in the gray matter of hippocampal slices (Wallraff et al. 2006). They

did not address whether the loss of both connexins affected K^+ buffering in other gray matter or myelinated regions of the CNS (see below). Even more surprisingly, they did not report an abnormal CNS phenotype for the mice that were deficient for both Cx30 and Cx43, as one might have predicted from the phenotype of mice lacking both Cx32 and Cx47.

If GJs mediate K^+ buffering, then O/A junctions are probably also important. The K^+ released from myelinated axons likely accumulates in the periaxonal space, where it may be dispersed by entering axons and oligodendrocytes via Na,K-ATPases (see below; Ransom et al. 2000) or possibly by leaking out via paranodal axoglial junctions (MacKenzie et al. 1984). Once K^+ enters the inner aspect of an oligodendrocyte, it may diffuse via reflexive Cx32/Cx32 channels and then enter astrocytes via O/A (Cx32/Cx30 and Cx47/Cx43) channels (see Fig. 7).

In addition to spatial buffering, glial cells remove extracellular K^+ by net K^+ uptake (Orkand 1986; Walz 2000; Kofuji and Newman 2004). Na,K-ATPases and ion cotransporters mediate intracellular uptake of extracellular K^+. In the rat optic nerve, inhibiting Na,K-ATPases interferes with the removal of extracellular K^+ (Ransom et al. 2000). Blocking Na–K–2Cl cotransport diminishes cell swelling in elevated extracellular K^+ (MacVicar et al. 2002). These mechanisms have been more closely studied astrocytes and Müller glial cells than in oligodendrocytes (Fink et al. 1996; Juhaszova and Blaustein 1997; MacVicar et al. 2002).

Inwardly rectifying K^+ (Kir) channels have been postulated to mediate the dispersal of K^+. Both astrocytes and oligodendrocytes express Kir channels, particularly Kir4.1 (Poopalasundaram et al. 2000; Neusch et al. 2001; Connors et al. 2004; Guadagno and Moukhles 2004; Kalsi et al. 2004; Kofuji and Newman 2004). Because Kir4.1 is open at the resting membrane potential, it contributes substantially to the hyperpolarization of astrocytes and oligodendrocytes (Neusch et al. 2001; Olsen et al. 2006; Djukic et al. 2007). Although Kir4.1 channels on astrocyte endfeet could mediate K^+ efflux and/or vasodilation (Kofuji et al. 2000; Higashi et al. 2001; Kofuji and Newman 2004), a recent study indicates that Kir4.1 is not required for vasodilation (Metea et al. 2007).

It is possible that local increases in K^+ could enter glial cells via Kir4.1 and then be redistributed by the GJs that extensively couple glial cells (Poopalasundaram et al. 2000). The vacuolated myelin in mice lacking Kir4.1 in all cells (Neusch et al. 2001; Djukic et al. 2007) provides indirect evidence that Kir4.1 plays a role in ion homeostasis. Furthermore, Menichella et al. (2006) provided genetic evidence that Kir4.1 and oligodendrocytes connexins interact: haploinsufficiency of both Cx47 and Kir4.1 in a *Gjb1/cx32*-null background resulted in widespread vacuolation of myelin, suggesting that oligodendrocyte connexins and Kir4.1 channels share a common pathway, the details of which remain to be shown.

Other Possible Roles of A/A and A/O Coupling

In addition to K^+ spatial buffering, GJs have also been postulated to play a role in the propagation of Ca^{2+} waves. This was initially proposed by Cornell-Bell et al. (1990), who showed that glutamate applied to confluent cultures of primary astrocytes induced oscillations of intracellular Ca^{2+} that appeared to propagate to neighboring cells (Cornell-Bell et al. 1990; Cornell-Bell and Finkbeiner 1991). A variety of stimuli can evoke such Ca^{2+} waves, including extracellular ATP or glutamate, the intracellular uncaging of IP_3, as well as electrical or mechanical stimulation (Charles et al. 1991; Dani et al. 1992; Newman and Zahs 1997; Fry et al. 2001). Two nonmutually exclusive pathways could mediate propagation of these waves—direct intercellular propagation (presumably of IP_3) mediated by GJs and extracellular propagation mediated by ATP released from stimulated cells (Scemes and Giaume 2006). Because Ca^{2+} waves can be elicited in nonadjacent cells, GJs are not absolutely required (Hassinger et al. 1996; Zanotti and Charles 1997; Charles 1998). Furthermore, the majority of studies implicating GJs (or hemichannels) in propagating Ca^{2+} waves relied on nonspecific blockers, such as heptanol, octanol, and carbenoxolone (Finkbeiner 1992; Nedergaard 1994; Guan et al. 1997; Naus et al. 1997; Stout et al. 2002). More recent studies have demonstrated that these compounds also block $P2X_7$ receptors, which respond to ATP (Suadicani et al. 2006). $P2X_7$ receptor activation has been reported to release ATP via pannexin1 hemichannels (Pelegrin and Surprenant 2006). However, in acute brain slices, the lack of Cx43 eliminates Ca^{2+} waves in the neocortex, indicating that GJs may be required for propagating Ca^{2+} waves in at least some brain regions (Haas et al. 2006).

Testing the types of signals that permeate O/A and A/A channels may help to determine whether they have an important role in activity-induced phenomena, such as spatial K^+ buffering or Ca^{2+} waves. To date, the endogenous signals that permeate GJ channels in general and those particular to specific tissues/cell types have remained elusive (Harris 2007). The size and shape of the permeating species and the molecular architecture of the channel pore, but not its unitary conductance, appear to influence permeability (Harris 2007). It is not surprising, then, that different connexin channels, such as Cx32/Cx32 and Cx43/Cx43, exhibit several-fold differences in permeability to molecules such as cAMP, glucose, glutamate, and glutathione (Goldberg et al. 2002; Bedner et al. 2006). Identifying small molecule candidates, such as IP_3 (Niessen et al. 2000) and ATP

(Goldberg et al. 2002), for each of the O/A (Cx47/Cx43 and Cx32/Cx30) and A/A (Cx30/Cx30 and Cx43/Cx43) channels we have described (Orthmann-Murphy et al. 2007b) may ultimately be useful. For example, one recent study found that ATP released from axons mediates the release of LIF from astrocytes, which in turn, induces myelination in mature oligodendrocytes (Ishibashi et al. 2006); perhaps, O/A channels propagate one or more of the signals that underlies this pathway.

Finally, it is possible that oligodendrocyte and astrocyte connexins have roles besides forming functional GJ channels. Cx43 can bind to many proteins involved in downstream cell signaling pathways, including various kinases and phosphatases (Giepmans 2004); it remains to be shown whether these interactions are required for signaling within the astrocyte. Findings from two recent studies suggest that the expression of the appropriate connexins, but not functional GJ formation per se, is required for neuronal migration in the cortex (Cx26 and Cx43; Elias et al. 2007) and lens differentiation (Cx45.6, the chick ortholog of Cx50; Banks et al. 2007). Thus, it is plausible that the interactions of oligodendrocyte and astrocyte connexins may have other functions and that alterations in these functions may play a role in the pathogenesis of PMLD, CMT1X, or ODDD.

Conclusion

The analysis of mutant mice and humans with PMLD, CMT1X, and ODDD provides compelling evidence that oligodendrocyte and astrocyte connexins are essential for the proper functioning of myelinated axons. The localization of specific connexins to O/A and A/A junctions, together with their compatibility in model systems, leads to the conclusion that O/A junctions are comprised of Cx32/Cx30 and Cx47/Cx43 channels and that A/A junctions are comprised of Cx30/Cx30 and Cx43/Cx43 channels.

Much remains to be learned. In particular, until the physiological roles of A/A and O/A channels are clarified, one cannot deduce the adaptive roles of the individual channels. There is ample reason to suspect a link between neuronal activity and the function of the "glial syncytium," even to the level of local populations of neurons (Halassa et al. 2007) and axons. Understanding of the function of O/A and A/A channels will help us more fully understand the underlying mechanism of disease for PMLD, CMT1X, and ODDD, leading to treatments for patients affected by these and other disorders.

Acknowledgment This work was supported by the National Multiple Sclerosis Society and the following NIH grants: NS054363 (to J.L.O.-M.), NS55284 and NS043560 (to S.S.S.), and NS50345 and NS050705 (to C.K.A.). We thank Dr. Bruce Altevogt, Dr. Michael Bennett, Dr. Kleopas Kleopa, Dr. Michael Koval, Dr. Daniela Menichella, Dr. David Paul, and Dr. Sabrina Yum for their many informative discussions.

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