Published in final edited form as:

Glia. 2011 July ; 59(7): 1064–1074. doi:10.1002/glia.21179.

Deletion of oligodendrocyte Cx32 and astrocyte Cx43 causes white matter vacuolation, astrocyte loss and early mortality

Laura M. Magnotti¹, Daniel A. Goodenough², and David L. Paul¹

¹Department of Neurobiology, Harvard Medical School, 220 Longwood Ave., Boston, MA 02115

²Department of Cell Biology, Harvard Medical School, 220 Longwood Ave., Boston, MA 02115

Abstract

CNS glia exhibit a variety of gap junctional interactions: between neighboring astrocytes, between neighboring oligodendrocytes, between astrocytes and oligodendrocytes, and as ‘reflexive’ structures between layers of myelin in oligodendrocytes. Together, these junctions are thought to form a network facilitating absorption and removal of extracellular K⁺ released during neuronal activity. In mice, loss of the two major oligodendrocyte connexins causes severe demyelination and early mortality, while loss of the two major astrocyte connexins causes mild dysmyelination and sensorimotor impairment, suggesting that reflexive and/or oligo-oligo coupling may be more important for the maintenance of myelin than other forms. To further explore the functional relationships between glial connexins, we generated double knockout mice lacking one oligodendrocyte and one astrocyte connexin. Cx32-Cx43 dKO animals develop white matter vacuolation without obvious ultrastructural abnormalities in myelin. Progressive loss of astrocytes but not oligodendrocytes or microglia accompanies sensorimotor impairment, seizure activity and early mortality at around 16 weeks of age. Our data reveal an unexpected role for connexins in the survival of white matter astrocytes, requiring the expression of particular isoforms in both oligodendrocytes and astrocytes.

Keywords

Gap junction; connexin; myelin; astrocyte; oligodendrocytes; Cx47; Cx32; Cx43

Introduction

CNS glia are extensively interconnected by gap junctions that allow the direct exchange of small molecules between adjacent cells. Gap junctions have been observed *in vivo* between neighboring astrocytes (A-A) and between astrocytes and oligodendrocytes (A-O) (Massa and Mugnaini, 1982). In addition, although there are no *in vivo* anatomical demonstrations of junctions between neighboring oligodendrocytes (O-O), junctional coupling assessed by dye transfer has been demonstrated (Maglione et al., 2010). Furthermore, individual oligodendrocytes establish ‘reflexive’ or ‘autologous’ gap junctions between layers of myelin and between successive paranodal loops (Kamasawa et al., 2005) that are believed to significantly shorten the distance for diffusion from periaxonal cytoplasm to oligodendrocyte soma. Thus, at least four types of junctional interactions can be established by CNS glial cells. Together, these junctions are thought to link cells into a network where at least one function is the absorption and removal of extracellular K⁺ released during

neuronal activity (Orkand et al., 1966; Kettenmann and Ransom, 1988; Menichella et al., 2006). The overall significance of glial gap junctions is underscored by the association of mutations in several human glial connexins with white matter abnormalities (Taylor et al., 2003; Loddenkemper et al., 2002; Uhlenberg et al., 2004). However, the relative importance of each type of junctional interaction is not clear.

Gap junction channels are composed of connexins, a family of >20 members in mammals (Goodenough and Paul, 2009). Oligodendrocytes express three connexins; Cx29, Cx32 and Cx47 (Altevogt et al., 2002; Scherer et al., 1995; Menichella et al., 2003; Odermatt et al., 2003) although Cx29 is not believed to participate in the formation of gap junctions (Altevogt et al., 2002; Ahn et al., 2008). Astrocytes express a different set of three; Cx26, Cx30 and Cx43 (Nagy et al., 2001; Kunzelmann et al., 1999; Giaume et al., 1991) although Cx26 displays strong regional differences in expression (Mercier and Hatton, 2001) and there is no clear consensus regarding its contributions to glial gap junctions (Filippov et al., 2003). As oligodendrocytes and astrocytes each express a distinct profile of connexins, gap junctions between these different cell types must be composed of heterotypic intercellular channels. Since Cx43 and Cx32 have been shown not to interact in several experimental systems (Elfgang et al., 1995; Orthmann-Murphy et al., 2007; Magnotti et al., 2010) it follows that most A-O intercellular channels contain heterotypic pairing of Cx30-Cx47, Cx43-Cx47, Cx26-Cx32 and/or Cx30-Cx32.

The CNS phenotypes of mice lacking connexins are summarized in Table 1. Mice lacking Cx32, Cx47, Cx43 or Cx30 display relatively subtle glial phenotypes, if any. However, loss of both major oligodendrocyte connexins (Cx47 and Cx32 = OO dKO) results in severe demyelination, massive apoptotic oligodendrocyte death and early mortality (Menichella et al., 2003). Loss of the two major astrocyte connexins (Cx43 and Cx30 = AA dKO), causes mild dysmyelination and modest sensorimotor impairment with no effect on longevity (Lutz et al., 2009). Intracellular injection of biocytin into oligodendrocyte cell bodies in the corpus callosum reveals that O-O and A-O coupling are lost in the OO dKO while A-A and A-O coupling are lost in the AA dKO (Maglione et al. 2010). Although reflexive coupling could not be assessed, the severity of the OO dKO phenotype relative to the AA dKO suggests that O-O and/or reflexive coupling are more critical for glial cell health and survival than other forms. To further explore the functional relationships of glial connexins, we constructed dKOs lacking one oligodendrocyte (Cx47 or Cx32) and one astrocyte (Cx43) connexin. Models based on connexin distribution *in vivo* and patterns of connexin interaction *in vitro* predict that Cx47-Cx43 and Cx32-Cx43 dKOs should exhibit at most only subtle behavioral abnormalities, similar to single KOs of each connexin. While the Cx47-Cx43 dKO phenotype was weak as predicted, Cx32-Cx43 dKOs displayed white matter vacuolation, sensorimotor impairment, absence-like seizure activity and early mortality with a median age of 16 weeks. Surprisingly, the onset of vacuolation was not accompanied by abnormalities in myelin ultrastructure that characterize the other double knockouts. At 16 weeks, the numbers of oligodendrocytes and microglia were normal but astrocytes were reduced by 25%. These features are not easily modeled by abnormalities in K⁺, recycling, as suggested for the other dKOs (Menichella et al., 2006; Wallraff et al., 2006; Lutz et al., 2009). Instead, our data indicate that connexins have an unexpected role in the maintenance of white matter astrocytes, which requires the expression of specific connexin genes in both astrocytes and oligodendrocytes.

Results

To generate double knockout mice lacking one oligodendrocyte (Cx47 or Cx32) and one astrocyte (Cx43) connexin, we crossed mice having a constitutive deletion of Cx32 (Nelles et al., 1996) or Cx47 (Menichella et al., 2003) with mice harboring two floxed alleles of

Cx43 (Cx43^{flx/flx}; Theis et al., 2001) and mice carrying the hGFAP-Cre transgene (Zhuo et al., 2001). Astrocyte-directed deletion of Cx43 was necessary since constitutive Cx43 KO mice are perinatal lethal (Reaume et al., 1995).

Cx32-Cx43 double knockouts display seizure-like activity, early mortality and motor impairment

OO dKOs, lacking both major oligodendrocyte connexins, display a coarse-action tremor and tonic-clonic seizures by the third postnatal week leading to early mortality between the 5th and 6th postnatal week (Menichella et al., 2003). AA dKOs, lacking both major astrocyte connexins, are not subject to seizure activity and live normal spans but as adults display impaired performance in motor tasks (Lutz et al., 2009). Therefore, we examined the Cx47-Cx43 and Cx32-Cx43 dKOs for similar behavioral abnormalities. No difference between Cx47-Cx43 dKOs and heterozygote and WT controls was noted (data not shown). However, Cx32-Cx43 dKOs consistently exhibited seizure-like activity. Initially, seizures were characterized by a short (5-30 second) behavioral arrest with occasional atonia, suggesting an absence or petit mal seizure. Subsequently, most animals developed tonic-clonic seizures. To determine the penetrance and onset, 30 dKO and 30 littermate controls were monitored for ~1hr/day between the ages of 6 and 16 weeks (Fig. 1A). No seizure-like activity was observed in dKOs before the 8th postnatal week but by the 11th postnatal week, >90% were affected. Thus, onset was relatively sharp and penetrance was nearly full. The Cx32-Cx43 dKOs also died prematurely. To evaluate this parameter, a cohort of 50 dKOs and 50 littermate controls was followed for 65 weeks by which time all of the mutants, but only two of the controls, had expired (Fig. 1B). Significant variability in the age of mortality was observed but >85% had died by the 20th postnatal week.

The effect of the Cx32-Cx43 dKO on motor coordination was evaluated in the balance beam task. Tests were performed on the same cohort of animals at 4, 6 and 8 weeks of age, well before the onset of seizure activity that might affect the results. As shown in the clustered multiple comparison graph (Fig. 1C), no difference in the mean rate of foot slippage between dKOs (green bar) and controls (yellow bar) was observed in 4 week old animals. However, a significant difference was observed at 6 weeks, which was strengthened at 8 weeks. Thus, motor impairment generally preceded the onset of seizures. However, the comparison revealed a surprising amount of variation in the performance of individuals, which might be explained by variation in the extent of recombination of the floxed Cx43 allele. To assess this, we evaluated Cx43 expression using immunohistochemistry on the same animals used in the balance beam study after the 8 week data had been collected (representative images shown in Fig. 1D-G). At low magnification, WT animals exhibited extensive Cx43 immunoreactivity in cerebellar white and gray matter (Fig. 1D). In a typical dKO (Fig. 1E), small islands of immunoreactivity were observed, usually less than 5% of the total area. However, a significantly larger area of Cx43 expression was observed in the best-performing individual in the balance beam test (Fig. 1F), while almost no Cx43 was evident in the worst-performing individual (Fig. 1G). All 10 dKO animals tested on the balance beam were examined for Cx43 expression and similar patterns of immunoreactivity were seen in the cerebellum and other CNS areas including the spinal cord. Overall we found a good correlation between motor performance and Cx43 expression in the CNS.

To determine if these differences reflected variation in the timing of Cx43 deletion, we performed a similar analysis on 2 week old mice (data not shown). We found comparable patterns of residual Cx43 expression in both 2 week and 8 week old animals. Taken together, these data suggest that hGFAP-Cre penetrance was variable in our line but that if recombination occurred, it did so largely before postnatal day 14. In addition, it was likely

that differences in Cx43 expression among individuals contributed to the observed differences in motor performance.

Double knockouts display vacuolation associated with white matter tracts

Since seizures, motor impairment, and early mortality were all consistent with an effect on myelin homeostasis as reported for OO (Menichella et al., 2003) and AA (Lutz et al., 2009) dKOs, we examined brain and optic nerve for white matter abnormalities. No abnormalities were observed for the Cx47-Cx43 dKO (data not shown). However, comparison of sagittal paraffin sections of 14 week old Cx32-Cx43 dKO and control brains (Fig.2) revealed marked vacuolation associated with white matter in corpus callosum (Fig.2B,E), anterior commissure (Fig.2H) and cerebellum (Fig. 2K) but not optic nerve (Fig.2N). No obvious gray matter abnormalities were observed. At the light microscopic level, Cx32-Cx43 dKO vacuolation was grossly similar in appearance to that found in the OO dKO (Fig. 2 C,F,I,L,O). However, the OO dKO vacuolation has a much earlier onset and was far more extensive than vacuolation in the Cx32-Cx43 dKO. In addition, OO dKO vacuolation was evident in optic nerve (Fig. 2O) and gray matter areas, which were not seen in the Cx32-Cx43 dKO. It has been reported that conditional deletion of Cx43 by hGFAP-Cre alone can cause abnormal development and cellular organization of the cerebellum, cortex, and hippocampus as well as behavioral abnormalities (Wiencken-Barger et al., 2007). However, we did not observe any gross patterning defect in single or double KOs.

To determine the developmental timing of vacuolation, brains were obtained from animals 2, 4, 5, 6, 12 and 14 weeks of age and sagittal paraffin sections were prepared. Representative sections of cerebellum are displayed in Fig. 3. Vacuolation appeared abruptly between the 4th and 5th postnatal week. Surprisingly, the extent of vacuolation did not increase in an obvious way between the 5th and 12th postnatal week. Similar timing of vacuolation was observed in corpus callosum and anterior commissure (data not shown). Thus, onset of vacuolation was extremely sharp.

Electron microscopy reveals that myelin is only modestly affected

To define the ultrastructural features of the pathology, areas of the deep cerebellar white matter and anterior commissure were studied by electron microscopy in 6-week-old animals, after the onset of vacuolation but well before seizures become evident. Large empty spaces (vacuoles), sometimes filled with debris, were frequently observed (Fig. 4A,B; *). However, most myelinated fibers were normal in appearance. Normal astrocytes (Fig. 4C; "A") were distinguished by a thin, uniform layer of condensed chromatin, the presence of intermediate filaments and a relatively electron-lucent cytoplasm (Vaughan and Peters, 1974) and were similar in appearance to controls (data not shown). Similarly, normal oligodendrocytes (Fig. 4D, "O") were identified by clumped heterochromatin, lack of intermediate filaments, and relatively electron-dense cytoplasm (Peters et al., 1976). However, what appeared to be empty sheaths with thinned myelin, suggesting axonal loss, were occasionally observed (Fig. 4D). More commonly, cellular processes exhibiting a washed-out and disorganized cytoplasm with fragmented organelles and vesicular inclusions were detected (Fig. 4D,E; ■). Cell bodies with similar features were often encountered (Fig. 4E; ▲) but the obvious pathology precluded firm identification. However, severely pathological cells exhibiting intermediate filaments, suggesting an astrocytic origin, were occasionally observed (Fig. 4F,G). Here, myelinated fibers appear to be surrounded by cytoplasm of the pathologic cell because its plasma membrane has been disrupted. Similarly, the cytoplasm seems to surround a blood vessel (BV), which is lacking any evidence of astrocyte endfeet. (G) A higher magnification of the boxed region in F reveals the presence of intermediate filaments. These pathologic cells never displayed morphological signs of apoptosis. Nuclear

condensation characteristic of apoptotic cell loss was never observed. Finally, vacuoles, empty spaces and cell bodies/processes with the characteristic washed-out and disorganized cytoplasm were never observed in litter mate control animals (data not shown).

Thus, Cx32-Cx43 dKO and OO dKO pathologies are very different. Electron microscopy reveals that the Cx32-Cx43 dKO exhibits largely healthy myelin, largely healthy oligodendrocytes and pathologic cells which are likely of astrocytic origin while the OO dKO is characterized by thinner or absent myelin sheaths, separation of axons from their myelin sheaths by a markedly enlarged extracellular space and apoptotic cells with ultrastructural characteristics suggestive of an oligodendrocyte origin (Menichella et al., 2004).

The Cx32-Cx43 dKO displays a selective loss of astrocytes but not oligodendrocytes

If the ultrastructural abnormalities reflect pathological changes in a particular glial cell type, then we might expect to find a decrease in number over time. Therefore, we performed a morphometric analysis to establish glial cell numbers at 6, 12, 14 and 17 weeks of age. To count mature oligodendrocytes, we stained sections of cerebellum for CC1 (Bhat et al., 1996) and used a Neurolucida workstation to count stained cells within defined areas of white matter (Fig. 5A). As shown in Fig. 5B, there was no significant difference between dKO and littermate controls at any time point. However, a slight downward trend in dKO numbers was noted at 14 and 17 weeks, perhaps not surprising as the median age of death was 16 weeks. To count activated microglia, we stained for the calcium binding protein Iba1 (Schluesener et al., 1998). A significant difference between dKO and control was only observed at 17 weeks (Fig. 5D), consistent with a morbidity involving neuro-inflammation but not the morphological abnormalities observed at early time points. To count astrocytes, we stained for Cre recombinase (Fig 5E). Most astrocytes in both dKO and controls expressed Cre recombinase, which resided largely in the nucleus and was easier to score than cytosolic markers like GFAP. Although the hGFAP-Cre line used in our studies has been reported to express Cre recombinase in neurons as well as astrocytes (Zhuo et al., 2001), this would not affect our results because we only counted nuclei in large white matter tracts. As shown in Fig. 5F, the dKO exhibited significant astrocyte loss relative to controls at 14 and 17 weeks as well as a downward trend at 12 weeks. Thus, the Cx32-Cx43 dKO pathology in the cerebellum featured a selective and progressive loss of astrocytes beginning between 6 and 12 weeks.

To test the possibility that astrocytes in the Cx32-Cx43 dKO mice might be undergoing apoptosis, we performed TUNEL staining for fragmented DNA on 6, 12, 14, and 17 week old animals. No obvious increase in apoptosis was seen in dKO brains at any age (data not shown). We then addressed the possibility that loss of astrocytes would affect the integrity of the blood-brain barrier (BBB), as astrocytes play a role in regulating its formation and maintenance. Mice were perfused with a solution containing the Evans Blue dye, which cannot normally pass through the BBB. At both 6 and 12 weeks of age, the Evans Blue was restricted to blood vessels in all animals, indicating that the BBB remained intact in the dKO animals (data not shown).

Discussion

We have shown that animals lacking both oligodendrocyte Cx32 and astrocyte Cx43 displayed seizures, motor impairment and early mortality. White matter vacuolation appeared abruptly at the 5th postnatal week but myelin ultrastructure was generally normal at this time. Instead, the earliest signs of pathology were restricted to astrocytes, which were

selectively lost. Together, these observations indicate an unexpected role for specific glial connexins in astrocyte survival.

It is surprising that the loss of astrocyte Cx43 and Cx30 (Lutz et al., 2009) was less deleterious than loss of astrocyte Cx43 and oligodendrocyte Cx32, as we report here. In the AA dKO, A-O coupling is eliminated, at least in corpus callosum (Maglione et al., 2010) and A-A coupling is likely to be dramatically reduced in most astrocytes (Theis et al., 2004). Yet, despite the possible loss of both A-A and A-O coupling, animals live a normal span. In contrast, it has been reported that A-O and O-O coupling were both unaffected in single Cx43 and Cx32 KOs (Maglione et al., 2010). Coupling patterns in the Cx32-Cx43 dKO have not been determined but since these connexins do not directly interact (Orthmann-Murphy et al., 2007), there is no obvious reason why A-O or O-O coupling would be more affected in the dKO than in either of the single KOs. Thus, paradoxically, what seems to be the more severe glial communication defect is associated with the weaker behavioral phenotype.

One explanation would be that a partial loss in reflexive coupling, due in this case to loss of Cx32, has severe consequences when combined with a partial reduction in some other types of coupling, due in this case to loss of Cx43. Freeze-fracture immuno-labeling microscopy indicates that Cx32 is more abundant than Cx47 at reflexive junctions while the opposite holds at A-O junctions (Kamasawa et al., 2005), suggesting that reflexive coupling would be more strongly affected by the loss of Cx32 and A-O coupling would be more strongly affected by loss of Cx47. Subsequently, it was shown using biocytin injection of corpus callosal oligodendrocytes that A-O and O-O coupling were indeed unaffected by loss of Cx32, while both were strongly affected by loss of Cx47 (Maglione et al., 2010). While reflexive coupling could not be directly measured, these observations support the idea that changes in reflexive coupling might be an important part of the Cx32-Cx43 dKO pathology. If that were true, then astrocyte-specific losses would be predicted in the OO dKO, where they were not observed. However, astrocyte-specific pathologies might be missed simply because the OO dKOs die at a much earlier stage. Furthermore, the notion that changes in reflexive coupling influence the Cx32-Cx43 dKO pathology is consistent with our observation of normal spans and lack of widespread white matter vacuolation in the Cx47-Cx43 dKO, where reflexive coupling should be only modestly affected.

An alternative possibility is that strain background strongly influences the phenotype. The AA dKO reported by Lutz et al. (2009) was extensively backcrossed to C57BL/6 while our dKOs were a mix of 129sv, C57BL/6 and FVB strains. Significant differences in phenotype relating to strain background have been reported for a number of genes (Gerlai, 1996) including a lens fiber connexin (Gerido et al., 2003). However, previous studies of a partially backcrossed AA dKO reported normal spans with no behavioral abnormalities (Wallraff et al., 2006). Furthermore, considerations of strain background would not invalidate a comparison between Cx32-Cx43 and OO dKOs (Menichella et al., 2003). While there are similar overall effects on behavior, the primary pathologies are very different. These observations strongly suggest that different types of coupling have different functions in glial homeostasis but that all may be critically important.

A difficulty for interpretation of available KO studies is determining the status of the various forms of junctional communication. First, there is no straightforward method to test reflexive coupling. Second, while cell-cell communication in CNS glia has been measured *in vivo* using microelectrode-based techniques, there are conflicting reports regarding the extent, or even the existence of A-O and O-O coupling (Ransom and Kettenmann, 1990; Robinson et al., 1993; Pastor et al., 1998; Alvarez-Maubecin et al., 2000; Houades et al., 2008; Maglione et al., 2010). Most recently, Maglione et al. (2010) evaluated glial cell communication in a number of connexin KO lines. They found A-O coupling was

unaffected in Cx43 KO corpus callosum but was eliminated in both Cx47 KO and Cx43-Cx30 (AA) dKO, implying that A-O coupling required heterotypic channels composed of astrocyte Cx30 and oligodendrocyte Cx47. Their data are consistent with the report that localization of Cx43, Cx30 and Cx32 in gap junctions at oligodendrocyte cell bodies is lost in Cx47 KO cortex, thalamus and brainstem (Li et al., 2008) and with the ability of Cx47 and Cx30 to form heterotypic channels when expressed in HeLa cells (Magnotti et al., 2011 but see Orthmann-Murphy et al., 2007). In contrast, the localization of Cx30 to puncta on spinal cord oligodendrocyte cell bodies was lost in the Cx32 KO while localization of Cx43 and Cx47 were unaffected (Altevogt and Paul, 2004), implying a heterotypic association of Cx30 and Cx32. The simplest interpretation of these data is the existence of two types of A-O heterotypic channels, the first composed of Cx43 and Cx47 and the second of Cx30 and Cx32. Regional and/or temporal variations in connexin distribution might underlie the apparent conflicts in the literature. Thus, glial communication might be specifically tailored to meet regional needs.

The canonical role for glial gap junctions is the spatial buffering of K^+ released during neuronal activity (Orkand et al., 1966). Astrocytes are highly permeable to K^+ and coupling between astrocytes could provide a mechanism for redistributing extracellular K^+ (Kofuji and Newman, 2004). In support of this idea, K^+ buffering was modestly affected in AA dKO mice (Wallraff et al., 2006). Studies of AA dKO mice also revealed that gap junctions between astrocytes allow an activity-dependent intercellular trafficking of metabolites necessary for sustained synaptic activity (Rouach et al., 2008). However, AA dKOs live normal spans. Thus, the contributions of A-A coupling to K^+ buffering or metabolic support for neurons are not critical for survival. In contrast, OO dKOs die early, indicating that expression of at least one oligodendrocyte connexin is fundamentally important. The similarity of the OO dKO phenotype to that of a Kir4.1 knockout (Neusch et al., 2001) led to the hypothesis that oligodendrocyte connexins may also contribute to K^+ buffering (Kamasawa et al., 2005);(Menichella et al., 2006) by allowing sequential movement of ions from the periaxonal cytoplasm to the oligodendrocyte cell body, through reflexive junctions, and then into the network of astrocytes through A-O junctions. Indeed, compound heterozygotes for Kir4.1 and oligodendrocyte connexins develop vacuolation similar to the OO dKO, suggesting that K^+ channels and connexins function in a common pathway. However, while both OO dKO and Cx32-Cx43 dKO both display seizures and early mortality, OO dKOs exhibit profound demyelination while the primary pathology in the Cx32-Cx43 dKO is astrocyte swelling and loss. It is not obvious why an interruption of the movement of K^+ from oligodendrocytes to astrocytes would result in astrocyte loss. Thus, we speculate that Cx32 and Cx43 together mediate signaling events promoting astrocyte survival. In support of this notion, it has been shown that exogenous expression of connexins can protect against certain forms of injury with a potency comparable to overexpression of bcl2 (Lin et al., 2003).. However, the nature of the signals and the details of the mechanism underlying astrocyte pathology in the Cx32-Cx43 dKO remain to be determined.

Methods and Materials

Animals

The generation of Cx43^{flx/flx} mice (Liao et al., 2001) and Cx32KO mice (Nelles et al., 1996) have been described previously. The hGFAP-Cre transgenic line was obtained from The Jackson Laboratory (Bar Harbor, ME). For most experiments, we compared mice lacking Cx32 globally and Cx43 in astrocytes (GFAP-Cre⁺, Cx43^{flx/flx}, Cx32KO) with littermate control mice lacking Cx32 but expressing one WT allele of Cx43 (GFAP-Cre⁺, Cx43^{flx/+}, Cx32KO). To produce those animals, we first crossed hGFAP-Cre⁺ males and Cx32KO females. Since Cx32 is X-linked, all male offspring from this crossing were hemizygous for

the Cx32 null allele. Cx32^{-/-}, hGFAP-Cre⁺ males were then crossed with females homozygous for the floxed allele of Cx43. Female offspring from this cross (Cx43^{+/-}, Cx32^{+/-}) were backcrossed to male parents to obtain breeding pairs for experiments consisting of GFAP-Cre⁻, Cx43^{flx/flx}, Cx32KO and GFAPCre⁺, Cx43^{+/-}, Cx32KO. Animals were maintained as approved by the Institutional Animal Care and Use Committee at Harvard Medical School.

Histology and electron microscopy

For histology, mice were anesthetized and perfused using Bouin's solution (5% acetic acid, 9% formaldehyde, 0.9% picric acid; Sigma). The entire mouse was submerged in fresh Bouin's solution for one week before brains and spinal cords were dissected. Tissue was then dehydrated and embedded in paraffin. Five micron thick sections were collected and slides were stained with Hematoxylin and Eosin (H and E). For ultrastructural studies, anesthetized mice were perfused with Karnovsky's fixative (3% glutaraldehyde, 3% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4) via the left cardiac ventricle. Tissue was isolated and post-fixed in fresh Karnovsky's overnight, then rinsed in PB, treated with 2% OsO₄ in 0.1M PB for 1 hr, dehydrated in graded ethanols, infiltrated, and embedded in epoxy. 70-nm sections were cut, stained with lead citrate and examined on a transmission electron microscope (1200EX; JEOL, Tokyo, Japan) at 80 kV and images recorded using digital plates.

Immunohistochemistry

Mice were anesthetized with 100 mg/ml ketamine (100 μ l per 10g body weight), then perfused using freshly prepared 1% paraformaldehyde in phosphate buffered saline (PBS). The spinal cord and brain were dissected and infiltrated in 30% sucrose in PBS overnight before embedding in Neg-50 (PerkinElmer, Waltham, MA). Cryosections (6 μ m) were mounted on SuperFrost Plus glass slides (VWR, West Chester, PA) and stored at -20°C. Sections were blocked with 10% fetal bovine serum and 0.2% Tween 20 in PBS for 30 minutes at room temperature. The sections were then incubated for one hour at room temperature with rabbit anti-Cx43 (1:10,000 dilution; Sigma-Aldrich, St Louis, MO). After three washes in PBS, sections were incubated for one hour with rhodamine-conjugated goat anti-rabbit (1:200; Chemicon, Temecula, CA). Nuclei were counter stained with DAPI (500ng/ml; Invitrogen). Slides were mounted with 50% PBS/50% glycerol and viewed on a Nikon E800 microscope (Tokyo, Japan) equipped with epifluorescence illumination (Prior Sci. Instr., Cambridge, UK) and a SPOT-RT slider digital camera (Diagnostic Instr., Sterling Heights, MI) was used. For cell counting experiments, sections were prepared as described above and stained with mouse anti-CC1 (APC (Ab-7), 1:20; Oncogene Research Products, Boston, MA), rabbit anti-glial fibrillary acidic protein (GFAP, 1:250 dilution; Dako, Carpinteria, CA), rabbit anti-Cre (1:500; Covance, Princeton, NJ) or rabbit anti-Iba1 (1:500; Wako Chemicals, Richmond, VA). After incubation with appropriate secondary antibodies, a morphometric analysis was performed in cerebellar white matter using Neurolucida software (MBF Bioscience, Williston, VT). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was performed on sectioned tissue prepared as described above according to the manufacturer's directions (Roche Diagnostics Corp., Indianapolis, IN).

Behavioral tests

All behavioral tests and scoring were performed blind to genotype. To determine the age of seizure onset, 30 pairs of mice (Cx32/GFAP-Cx43 mutant and littermate control) were observed daily for one hour at the same time each day beginning at 6 weeks of age and noting the total number of episodes. For the mortality study, 50 pairs of mice were followed and age of death was recorded until either both animals died or reached 65 weeks of age. Seizure onset and survival curves were plotted using the Prism 5 software (GraphPad

Software, La Jolla, CA) and significance was determined using the Gehan-Breslow-Wilcoxon test. For the balance beam test of motor coordination, mice underwent three 10 minute trials one hour apart to train them to walk on a wide (20 mm width $\times$ 1 m length) balance beam. At the end of the training trials, no freezing behavior was observed, and the mice would start to walk within 10 seconds of being placed on the beam. The mice were then videotaped as they performed two test trials of three beam walks each, one set of trials on the wide beam and another set on a narrow beam (4 mm width $\times$ 1 m length). The test trials were all performed on the same day. Videotaped walks were scored for number of foot slips (errors) and time to cross, and scoring was performed blind to genotype. Data are expressed as mean $\pm$ SEM and significance was evaluated using the unpaired one-tailed Student's *t* test. Differences among genotypes were considered significant if *p* < 0.05.

Measurement of Blood-Brain Barrier (BBB) integrity

This experimental design was adapted from del Valle et al. (del Valle et al., 2008). After animals were anesthetized, the thoracic cavity was opened and a 25ga butterfly, connected to a buffer reservoir 90 cm above the animal, was introduced into the left ventricle. Animals were then gravity perfused with 50mL of phosphate buffered saline (PBS, pH 7.2), followed by 50mL of a cocktail containing 1% Evans Blue (EB; Sigma-Aldrich), 0.01% Hoechst 33258 (H-33258, Invitrogen) and 4% paraformaldehyde (PFA; Electron Microscopy Sciences) in PBS. Thus, each mouse received both 500mg of EB as well as 5mg of Hoechst during the perfusion. Brains were removed, post-fixed with 4% PFA in PBS for 4 h and cryoprotected by immersion in PBS with 30% sucrose for 24 h before embedding in Neg-50. Twenty micron frozen sections were prepared and viewed on a Nikon E800 microscope.

Acknowledgments

This work was supported by NIH RO1 GM37751 to DLP, RO1 GM18974 to DAG, P30-HD18655 to the IDDRRC at Children's Hospital, Boston and P30-EY12196 to Harvard Medical School, Boston. We are grateful for the expert technical assistance of Heather Topley and Yaqiao Li.

Reference List

1. Ahn M, Lee J, Gustafsson A, Enriquez A, Lancaster E, Sul JY, Haydon PG, Paul DL, Huang Y, Abrams CK, Scherer SS. Cx29 and Cx32, two connexins expressed by myelinating glia, do not interact and are functionally distinct. *J Neurosci Res.* 2008; 86:992–1006. [PubMed: 17972320]
2. Altevogt BM, Kleopa KA, Postma FR, Scherer SS, Paul DL. Connexin29 is uniquely distributed within myelinating glial cells of the central and peripheral nervous systems. *J Neurosci.* 2002; 22:6458–6470. [PubMed: 12151525]
3. Altevogt BM, Paul DL. Four classes of intercellular channels between glial cells in the CNS. *J Neurosci.* 2004; 24:4313–4323. [PubMed: 15128845]
4. Alvarez-Maubecin V, Garcia-Hernandez F, Williams JT, Van Bockstaele EJ. Functional Coupling between Neurons and Glia. *J Neurosci.* 2000; 20:4091–4098. [PubMed: 10818144]
5. Bhat RV, Axt KJ, Fosnaugh JS, Smith KJ, Johnson KA, Hill DE, Kinzler KW, Baraban JM. Expression of the APC tumor suppressor protein in oligodendroglia. *Glia.* 1996; 17:169–174. [PubMed: 8776583]
6. del Valle J, Camins A, Pallas M, Vilaplana J, Pelegri C. A new method for determining blood-brain barrier integrity based on intracardiac perfusion of an Evans Blue-Hoechst cocktail. *J Neurosci Methods.* 2008; 174:42–49. [PubMed: 18647619]
7. Dere E, Souza-Silva MA, Frisch C, Teubner B, Sohl G, Willecke K, Huston JP. Connexin30-deficient mice show increased emotionality and decreased rearing activity in the open-field along with neurochemical changes. *Eur J Neurosci.* 2003; 18:629–638. [PubMed: 12911759]
8. Elf gang C, Eckert R, Lichtenberg-Fraté H, Butterweck A, Traub O, Klein RA, Hülser DF, Willecke K. Specific permeability and selective formation of gap junction channels in connexin-transfected HeLa cells. *J Cell Biol.* 1995; 129:805–817. [PubMed: 7537274]

9. Filippov MA, Hormuzdi SG, Fuchs EC, Monyer H. A reporter allele for investigating connexin 26 gene expression in the mouse brain. *Eur J Neurosci.* 2003; 18:3183–3192. [PubMed: 14686892]
10. Frisch C, Theis M, Souza Silva MA, Dere E, Sohl G, Teubner B, Namestkova K, Willecke K, Huston JP. Mice with astrocyte-directed inactivation of connexin43 exhibit increased exploratory behaviour, impaired motor capacities, and changes in brain acetylcholine levels. *Eur J Neurosci.* 2003; 18:2313–2318. [PubMed: 14622192]
11. Gabriel H-D, Jung D, Bützler C, Temme A, Traub O, Winterhager E, Willecke K. Transplacental uptake of glucose is decreased in embryonic lethal connexin26-deficient mice. *J Cell Biol.* 1998; 140:1453–1461. [PubMed: 9508777]
12. Gerido DA, Sellitto C, Li L, White TW. Genetic background influences cataractogenesis, but not lens growth deficiency, in Cx50-knockout mice. *Invest Ophthalmol Vis Sci.* 2003; 44:2669–2674. [PubMed: 12766071]
13. Gerlai R. Gene-targeting studies of mammalian behavior: is it the mutation or the background genotype? *Trends Neurosci.* 1996; 19:177–181. [PubMed: 8723200]
14. Giaume C, Fromaget C, El Aoumari A, Cordier J, Glowinski J, Gros D. Gap junctions in cultured astrocytes: single-channel currents and characterization of channel-forming protein. *Neuron.* 1991; 6:133–143. [PubMed: 1702648]
15. Goodenough DA, Paul DL. Gap junctions. *Cold Spring Harbor Perspect Biol.* 2009; 1:a002576.
16. Houades V, Koulakoff A, Ezan P, Seif I, Giaume C. Gap junction-mediated astrocytic networks in the mouse barrel cortex. *J Neurosci.* 2008; 28:5207–5217. [PubMed: 18480277]
17. Kamasawa N, Sik A, Morita M, Yasumura T, Davidson KG, Nagy JI, Rash JE. Connexin-47 and connexin-32 in gap junctions of oligodendrocyte somata, myelin sheaths, paranodal loops and Schmidt-Lanterman incisures: implications for ionic homeostasis and potassium siphoning. *Neuroscience.* 2005; 136:65–86. [PubMed: 16203097]
18. Kettenmann H, Ransom BR. Electrical coupling between astrocytes and between oligodendrocytes studied in mammalian cell cultures. *Glia.* 1988; 1:64–73. [PubMed: 2853139]
19. Kofuji P, Newman EA. Potassium buffering in the central nervous system. *Neuroscience.* 2004; 129:1045–1056. [PubMed: 15561419]
20. Kunze A, Congreso MR, Hartmann C, Wallraff-Beck A, Huttmann K, Bedner P, Requardt R, Seifert G, Redecker C, Willecke K, Hofmann A, Pfeifer A, Theis M, Steinhauser C. Connexin expression by radial glia-like cells is required for neurogenesis in the adult dentate gyrus. *Proc Natl Acad Sci U S A.* 2009; 106:11336–11341. [PubMed: 19549869]
21. Kunzelmann P, Schroder W, Traub O, Steinhauser C, Dermietzel R, Willecke K. Late onset and increasing expression of the gap junction protein connexin30 in adult murine brain and long-term cultured astrocytes. *Glia.* 1999; 25:111–119. [PubMed: 9890626]
22. Li X, Penes M, Odermatt B, Willecke K, Nagy JI. Ablation of Cx47 in transgenic mice leads to the loss of MUPP1, ZONAB and multiple connexins at oligodendrocyte-astrocyte gap junctions. *Eur J Neurosci.* 2008; 28:1503–1517. [PubMed: 18973575]
23. Liao Y, Day KH, Damon DN, Duling BR. Endothelial cell-specific knockout of connexin 43 causes hypotension and bradycardia in mice. *Proc Natl Acad Sci (USA).* 2001; 98:9989–9994. [PubMed: 11481448]
24. Lin JH, Yang J, Liu S, Takano T, Wang X, Gao Q, Willecke K, Nedergaard M. Connexin Mediates Gap Junction-Independent Resistance to Cellular Injury. *J Neurosci.* 2003; 23:430–441. [PubMed: 12533603]
25. Lodenkemper T, Grote K, Evers S, Oelerich M, Stogbauer F. Neurological manifestations of the oculodentodigital dysplasia syndrome. *J Neurol.* 2002; 249:584–595. [PubMed: 12021949]
26. Lutz SE, Zhao Y, Gulinello M, Lee SC, Raine CS, Brosnan CF. Deletion of astrocyte connexins 43 and 30 leads to a dysmyelinating phenotype and hippocampal CA1 vacuolation. *J Neurosci.* 2009; 29:7743–7752. [PubMed: 19535586]
27. Maglione M, Tress O, Haas B, Karam K, Trotter J, Willecke K, Kettenmann H. Oligodendrocytes in mouse corpus callosum are coupled via gap junction channels formed by connexin47 and connexin32. *Glia.* 2010; 58:1104–1117. [PubMed: 20468052]
28. Magnotti LM, Goodenough DA, Paul DL. Functional heterotypic interactions between astrocyte and oligodendrocyte connexins. *Glia.* 2010 in press.

29. Massa PT, Mugnaini E. Cell junctions and intramembrane particles of astrocytes and oligodendrocytes: a freeze-fracture study. *Neuroscience*. 1982; 7:523–538. [PubMed: 7078735]
30. Menichella DM, Goodenough DA, Sirkowski E, Scherer SS, Paul DL. Connexins are critical for normal myelination in the CNS. *J Neurosci*. 2003; 23:5963–5973. [PubMed: 12843301]
31. Menichella DM, Majdan M, Awatramani R, Goodenough DA, Sirkowski E, Scherer SS, Paul DL. Genetic and physiological evidence that oligodendrocyte gap junctions contribute to spatial buffering of potassium released during neuronal activity. *J Neurosci*. 2006; 26:10984–10991. [PubMed: 17065440]
32. Mercier F, Hatton GI. Connexin 26 and basic fibroblast growth factor are expressed primarily in the subpial and subependymal layers in adult brain parenchyma: Roles in stem cell proliferation and morphological plasticity? *J Comp Neurol*. 2001; 431:88–104. [PubMed: 11169992]
33. Nagy JI, Li X, Rempel J, Stelmack G, Patel D, Staines WA, Yasumura T, Rash JE. Connexin26 in adult rodent central nervous system: Demonstration at astrocytic gap junctions and colocalization with connexin30 and connexin43. *J Comp Neurol*. 2001; 441:302–323. [PubMed: 11745652]
34. Nakase T, Naus CC. Gap junctions and neurological disorders of the central nervous system. *Biochim Biophys Acta*. 2004; 1662:149–158. [PubMed: 15033585]
35. Nelles E, Bützler C, Jung D, Temme A, Gabriel H-D, Dahl U, Traub O, Stümpel F, Jungermann K, Zielasek J, Toyka KV, Dermietzel R, Willecke K. Defective propagation of signals generated by sympathetic nerve stimulation in the liver of connexin32-deficient mice. *Proc Natl Acad Sci (USA)*. 1996; 93:9565–9570. [PubMed: 8790370]
36. Neusch C, Rozengurt N, Jacobs RE, Lester HA, Kofuji P. Kir4.1 potassium channel subunit is crucial for oligodendrocyte development and in vivo myelination. *J Neurosci*. 2001; 21:5429–5438. [PubMed: 11466414]
37. Odermatt B, Wellershaus K, Wallraff A, Seifert G, Degen J, Euwens C, Fuss B, Bussow H, Schilling K, Steinhauser C, Willecke K. Connexin 47 (Cx47)-deficient mice with enhanced green fluorescent protein reporter gene reveal predominant oligodendrocytic expression of Cx47 and display vacuolized myelin in the CNS. *J Neurosci*. 2003; 23:4549–4559. [PubMed: 12805295]
38. Orkand RK, Nicholls JG, Kuffler SW. Effect of nerve impulses on the membrane potential of glial cells in the central nervous system of amphibia. *J Neurophysiol*. 1966; 29:788–806. [PubMed: 5966435]
39. Orthmann-Murphy JL, Freidin M, Fischer E, Scherer SS, Abrams CK. Two distinct heterotypic channels mediate gap junction coupling between astrocyte and oligodendrocyte connexins. *J Neurosci*. 2007; 27:13949–13957. [PubMed: 18094232]
40. Pastor A, Kremer M, Moller T, Kettenmann H, Dermietzel R. Dye coupling between spinal cord oligodendrocytes: differences in coupling efficiency between gray and white matter. *Glia*. 1998; 24:108–120. [PubMed: 9700494]
41. Peters, A.; Palay, S.; Webster, Hd. The fine structure of the nervous system: Neurons and their supporting cells. Saunders; Philadelphia: 1976.
42. Ransom BR, Kettenmann H. Electrical coupling, without dye coupling, between mammalian astrocytes and oligodendrocytes in cell culture. *Glia*. 1990; 3:258–266. [PubMed: 2144505]
43. Reaume AG, De Sousa PA, Kulkarni S, Langille BL, Zhu D, Davies TC, Juneja SC, Kidder GM, Rossant J. Cardiac malformation in neonatal mice lacking connexin43. *Science*. 1995; 267:1831–1834. [PubMed: 7892609]
44. Robinson SR, Hampson ECGM, Munro MN, Vaney DI. Unidirectional coupling of gap junctions between neuroglia. *Science*. 1993; 262:1072–1074. [PubMed: 8093125]
45. Rouach N, Koulakoff A, Abudara V, Willecke K, Giaume C. Astroglial metabolic networks sustain hippocampal synaptic transmission. *Science*. 2008; 322:1551–1555. [PubMed: 19056987]
46. Sargiannidou I, Vavlitou N, Aristodemou S, Hadjisavvas A, Kyriacou K, Scherer SS, Kleopa KA. Connexin32 mutations cause loss of function in Schwann cells and oligodendrocytes leading to PNS and CNS myelination defects. *J Neurosci*. 2009; 29:4736–4749. [PubMed: 19369543]
47. Scherer SS, Deschenes SM, Xu YT, Grinspan JB, Fischbeck KH, Paul DL. Connexin32 is a myelin-related protein in the PNS and CNS. *J Neurosci*. 1995; 15:8281–8294. [PubMed: 8613761]

48. Schluesener HJ, Seid K, Kretschmar J, Meyermann R. Allograft-inflammatory factor-1 in rat experimental autoimmune encephalomyelitis, neuritis, and uveitis: expression by activated macrophages and microglial cells. *Glia*. 1998; 24:244–251. [PubMed: 9728770]
49. Taylor RA, Simon EM, Marks HG, Scherer SS. The CNS phenotype of X-linked Charcot-Marie-Tooth disease: more than a peripheral problem. *Neurology*. 2003; 61:1475–1478. [PubMed: 14663027]
50. Theis M, Jauch R, Zhuo L, Speidel D, Wallraff A, Doring B, Frisch C, Sohl G, Teubner B, Euwens C, Huston J, Steinhauser C, Messing A, Heinemann U, Willecke K. Accelerated hippocampal spreading depression and enhanced locomotory activity in mice with astrocyte-directed inactivation of connexin43. *J Neurosci*. 2003; 23:766–776. [PubMed: 12574405]
51. Theis M, Speidel D, Willecke K. Astrocyte cultures from conditional connexin43-deficient mice. *Glia*. 2004; 46:130–141. [PubMed: 15042581]
52. Uhlenberg B, Schuelke M, Ruschendorf F, Ruf N, Kaindl AM, Henneke M, Thiele H, Stoltenberg-Didinger G, Aksu F, Topaloglu H, Nurnberg P, Hubner C, Weschke B, Gartner J. Mutations in the gene encoding gap junction protein alpha 12 (connexin 46.6) cause Pelizaeus-Merzbacher-like disease. *Am J Hum Genet*. 2004; 75
53. Vaughan DW, Peters A. Neuroglial cells in the cerebral cortex of rats from young adulthood to old age: an electron microscope study. *J Neurocytol*. 1974; 3:405–429. [PubMed: 4373545]
54. Wallraff A, Kohling R, Heinemann U, Theis M, Willecke K, Steinhauser C. The impact of astrocytic gap junctional coupling on potassium buffering in the hippocampus. *J Neurosci*. 2006; 26:5438–5447. [PubMed: 16707796]
55. Wiencken-Barger AE, Djukic B, Casper KB, McCarthy KD. A role for Connexin43 during neurodevelopment. *Glia*. 2007; 55:675–686. [PubMed: 17311295]
56. Zhuo L, Theis M, Alvarez-Maya I, Brenner M, Willecke K, Messing A. hGFAP-cre transgenic mice for manipulation of glial and neuronal function in vivo. *Genesis*. 2001; 31:85–94. [PubMed: 11668683]

Fig.1. Cx32-Cx43 dKOs display seizure-like activity, early mortality and motor impairment
 (A) No seizure-like activity was observed in dKOs before the 8th postnatal week but by the 11th postnatal week, >90% were affected. (B) Significant variability in the age of mortality was observed but >85% had died by the 20th postnatal week. By week 65, all of the mutants, but only two of the controls, had died. (C) Control and dKO animals performed equally well at the balance beam task at 4 weeks of age. A significant difference in the mean rate of foot slippage (green and yellow bars) was observed at 6 weeks which increased at 8 weeks although there were large differences in the performance of individuals. (D) WT animals exhibited extensive Cx43 immunoreactivity in cerebellar white and gray matter. (E) In a typical dKO, small islands of immunoreactivity were observed. A significantly larger area of Cx43 expression was observed in the best-performing individual (F) while almost no Cx43 was evident in the worst-performing individual (G). Scale bar: 100 μ m.

Fig 2. Cx32-Cx43 dKOs display white matter vacuolation

H&E-stained sagittal paraffin sections of 14 week old animals. The Cx32-Cx43 dKO shows gross vacuolation in the corpus callosum (B,E), anterior commissure (H) and cerebellar white matter (K) but not optic nerve (N). No gray matter vacuolation was noted. Littermate controls (Cre+, Cx43+/-, Cx32KO; A,D,G,J,M) are normal. Vacuolation is similar but less extensive than that found in Cx47-Cx32 (OO) dKO (C,F,I,L,O). Scale bar: 100 μ m.

Fig. 3. The onset of vacuolation occurs sharply between 4th and 5th postnatal week
H&E-stained sagittal sections of cerebellum from Cx32-Cx43 dKO and controls. Vacuoles are not observed at 4 weeks but are robust at 5 weeks. The extent of vacuolation increases only modestly from that point. Scale bar: 400 μ m.

Fig. 4. Electron microscopy reveals occasional empty sheaths with thinned myelin and glial cells with pathological features

(A,B) Large empty or debris-filled spaces (*) were frequently observed, although most myelinated fibers were normal in appearance. (C) Normal astrocytes ("A") were distinguished by a thin, uniform layer of condensed chromatin and a relatively electron-lucent cytoplasm. (D) Oligodendrocytes ("O"), identified by clumped heterochromatin, lack of intermediate filaments, and relatively electron-dense cytoplasm were generally normal in appearance but occasional empty sheaths with thinned myelin, indicating axonal loss, were detected. (E) Cellular processes exhibiting a disorganized cytoplasmic structure and vesicular inclusions (■) were commonly observed while cells with similar features were occasionally encountered (▲). (F) Occasionally, a severely pathological cell displaying intermediate filaments consistent with an astrocytic origin was detected. Pathology is indicated by the fragmentation of the plasma membrane, leaving myelinated fibers freely floating in the cytoplasm of the pathologic cell. In addition, a blood vessel surrounded by this cytoplasm but lacking astrocyte endfeet is evident. (G) A higher magnification of the boxed region in F is reveal the presence of intermediate filaments. Scale bars: 2 μ m (A-E), 500 nm (F-G).

Fig. 5. The Cx32-Cx43 dKO displays a selective loss of astrocytes but not oligodendrocytes Morphometric analysis established glial cell numbers at 6, 12, 14 and 17 weeks of age. (A) CC1 positive cells were counted in white matter of the cerebellum (outlined in white). (B) There was no significant difference in the number of CC1-positive oligodendrocytes between Cx32-Cx43 dKO and littermate controls at any time point. (C) Iba1 immunostaining labeled activated microglia in cerebellar white matter. (D) A significant difference in the number of Iba1-positive microglia was only observed at 17 weeks. (E) Cre staining labeled astrocytes and Bergmann glia. Only white matter Cre+ astrocytes were counted for this analysis. (F) The Cx32-Cx43 dKO exhibited significant loss of Cre-positive astrocytes relative to controls at 14 and 17 weeks as well as a downward trend at 12 weeks. Scale bar: 100 μ m.

Table 1

Genotype	CNS Glial expression	KO Phenotype related to CNS	Reference
Cx32 KO	Oligodendrocyte	Modest reduction in spinal cord myelin volume, no gross behavioral defect, normal span	(Sargiannidou et al., 2009)
Cx47 KO	Oligodendrocyte	Sporadic myelin vacuolation, no gross behavioral defect, normal span	(Menichella et al., 2003; Odermatt et al., 2003)
Cx26 KO	Astrocyte	Not tested due to embryonic lethality from failure of transplacental transport of glucose	(Gabriel et al., 1998)
Cx30 KO	Astrocyte	Increased 'emotionality', normal span	(Dere et al., 2003)
Cx43 KO	Astrocyte	Enhanced stroke volume after MCA occlusion, accelerated spreading depression, normal span	(Nakase and Naus, 2004; Theis et al., 2003; Frisch et al., 2003)
Cx30-Cx43 dKO	A-A	Elevated hippocampal [K+], more severe response to CNS ischemia, glial 'edema', myelin vacuolation, modest sensorimotor impairment, reduction in radial glial proliferation, normal span	(Wallraff et al., 2006; Lutz et al., 2009; Kunze et al., 2009)
Cx32-Cx47 dKO	O-O	Severe myelin vacuolation, axonal loss, oligodendrocyte cell death, action tremor, tonic-clonic seizure activity, early mortality.	(Menichella et al., 2003; Menichella et al., 2006; Odermatt et al., 2003)
Cx32-Cx43 dKO	O-A	Myelin vacuolation, astrocyte loss, absence and tonic-clonic seizure activity, early mortality	This study
Cx47-Cx43 dKO	O-A	No gross behavioral defect, normal span	This study